Exhibit A

D4

INSEMINATION OF MARES WITH LOW NUMBERS OF EITHER UNSEXED OR SEXED SPERMATOZOA

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ABSTRACT

Two experiments were conducted to determine pregnancy rates in mares inseminated 1) with 5, 25 and 500 x 10^6 progressively motile spermatozoa (pms), or 2) with 25 x 10^6 sex-sorted cells. In Experiment 1, mares were assigned to 1 of 3 treatments: Group 1 (n= 20) was inseminated into the uterine body with 500 x 10^6 pms. Group 2 (n=21) and Group 3 (n=20) were inseminated into the tip of the uterine horn ipsilateral to the preovulatory follicle with 25 and 5 x 10^6 pms, respectively. Mares in all 3 groups were inseminated either 40 (n=32) or 34 h (n=29) after GnRH administration.

More mares became pregnant when inseminated with 500 x 10^6 (18/20 = 90%) than with 25 x 10^6 pms (12/21 = 57%; P<0.05), but pregnancy rates were similar for mares inseminated with 25 x 10^6 vs 5 x 10^6 pms (7/20 = 35%) (P>0.1). In Experiment 2, mares were assigned to 1 of 2 treatments: Group A (n=11) was inseminated with 25 x 10^6 spermatozoa sorted into X and Y chromosome-bearing populations in a skimmilk extender. Group B (n=10) mares were inseminated similarly except that spermatozoa were sorted into the skimmilk extender + 4% egg yolk. Inseminations were performed 34 h after GnRH administration. Freshly collected semen was incubated in 224 µM Hoechst 33342 at 400 x 10^6 sperm/mL in HBGM-3 for 1 hr at 35°C and then diluted to 100 x 10^6 sperm/mL for sorting. Sperm were sorted by sex using flow cytometer/cell sorters. Spermatozoa were collected at approximately 900 cells/sec into either the extender alone (Group A) or extender + 4% egg yolk (Group B), centrifuged and suspended to 25 x 10^6 sperm/mL and immediately inseminated. Pregnancy rates were similar (P>0.1) between the sperm treatments (extender alone = 3/10, 30% vs 4% EY + extender = 2/10, 20%). Based on ultrasonography, fetal sex at 60 to 70 d correlated perfectly with the sex of the sperm inseminated, demonstrating that foals of predetermined sex can be obtained following nonsurgical insemination with sexed spermatozoa.

Key words: sexed spermatozoa, AI, equine, flow cytometry

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INTRODUCTION

Maximum fertility has been obtained in mares inseminated with 500 x 10^6 progressively motile spermatozoa (pms) every other day while the mares are in estrus (9). However, the minimum required number of motile spermatozoa for optimal fertility results in the mare has yet to be established, although 250 to 500 x 10^6 pms per insemination are usually recommended (27). Under ideal conditions, mares can be inseminated with as few as 100 x 10^6 pms every other day during estrus without lowering fertility (25). Insemination of mares with a low number of spermatozoa may be necessary when semen is limited or when using sexed spermatozoa. Currently, it is possible to sort about 1,000 live sperm/sec of each sex chromosomal composition by flow cytometry with 90% accuracy; thus, an insemination dose of 100 x 10^6 cells would require more than 24 h to sort.

Under natural mating conditions, most of the equine ejaculate is deposited directly into the uterus of the mare (8). This is also the case when performing routine AI. This deposition site is acceptable when high numbers of fertile spermatozoa are available for insemination, but when limited numbers are available, an alternative approach may be desired. Deep intrauterine insemination is a proven technique in cattle. Senger et al. (33) compared pregnancy rates when semen was deposited into the uterine body with those when deposited into both uterine horns (cornual insemination). Pregnancy rates for deposition into the uterine body were 43% compared with 65% for cornual insemination. The later method of insemination was also shown to be effective in cattle by Seidel et al. (36, 52). However, McKenna et al. (21) did not report an advantage using this technique (21).

Johnson et al. (15) used DNA as a marker to sort spermatozoa and reported subsequent birth of rabbits of the predicted sex following surgical insemination. Spermatozoa were separated into X and Y chromosome-bearing populations using a flow cytometry cell sorter. Sorted spermatozoa have been surgically inseminated into the uterus of rabbits (15) and swine (12). In vitro fertilization has been used to obtain pregnancies in cattle using sexed spermatozoa. Cran et al. (2) transferred 2 embryos into each of 9 heifers, 4 of these heifers became pregnant, and 6 calves were born, all of the predicted sex. This was followed by a larger field trial (3) in which 41 calves were born, with 37 being of the preselected sex. These results show that viable spermatozoa can be separated into X and Y chromosome-bearing populations and retain their capacity for fertilization and production of normal progeny. Pregnancies following in vitro fertilization with X and Y bearing spermatozoa have also been obtained in humans (18), swine (6, 19, 28) and rabbits (22). Cran et al. (4) demonstrated for the first time that pregnancy in sheep and subsequent birth of lambs of the predetermined sex could be obtained by laparoscopic AI of a low number of sorted sperm cells at the tip of the uterine horn. Pregnancies in cattle have also been produced following nonsurgical insemination of sexed spermatozoa (30, 32). More recently, Seidel et al. (31) obtained a 51% pregnancy rate in heifers inseminated with sexed, frozen spermatozoa. In the mare, 1 foal of the predicted sex has been produced in our laboratory following surgical insemination into the oviduct with 150,000 sexed spermatozoa.

The objective of our study was to compare pregnancy rates in mares inseminated on a single occasion close to ovulation, with 5, 25 or 500 x 10^6 pms and to determine if pregnancies would

result following nonsurgical insemination with 25 x 10^6 sexed sperm cells. Sorted spermatozoa were suspended either in a dried skimmilk extender or in a dried skimmilk extender + 4% egg yolk. The addition of egg yolk to the skimmilk extender had previously (10) been shown to improve spermatozoal motility in the stallion.

MATERIALS AND METHODS

Experiment 1. Unsexed Spermatozoa

Mares. Sixty-one reproductively normal, cyclic mares of light-horse breeds, ranging in age from 3 to 15 yr, were used from May 1 to July 31, 1998. Cloprostenol (250 µg, im) was administered to the mares to induce luteolysis, after which they were examined by palpation and ultrasonography of the reproductive tract per rectum every other day until a follicle >30 mm was detected, and then daily until ovulation. On the day of planned insemination, reproductive tracts were examined at a 12-h interval to determine the time of ovulation more precisely. Once a mare developed a follicle ≥35 mm, a GnRH analog implant (deslorelin acetate 2.2 mg, Ovuplant®, Fort Dodge, IA) was administered subcutaneously, and she was assigned to 1 of 3 treatment groups.

Treatment group 1. Mares were inseminated on a single occasion with 500 x 10^6 pms in 20 mL of dried skimmilk extender (EZ-Mixin® OF, Animal Reproduction Systems, Chino, CA) (25 x 10^6 pms/mL), either 40 h (n=9) or 34 h (n=11) after GnRH administration. Semen was deposited into the uterine body using a flexible plastic artificial insemination (AI) pipette (I.M.V.; L'Aigle, France).

Treatment group 2. Mares were inseminated on a single occasion with 25 x 10^6 pms in 1 mL of dried skimmilk extender (25 x 10^6 pms/mL), either 40 h (n=13) or 34 h (n=8) after GnRH administration. Semen was deposited at the tip of the uterine horn, ipsilateral to the preovulatory follicle, using a flexible plastic AI pipette. The location of pipette within the uterus was confirmed by transrectal ultrasonography prior to semen deposition.

Treatment group 3. Mares were inseminated on a single occasion with 5 x 10^6 pms in either 1 mL of dried skimmilk extender (5 x 10^6 pms/mL) or 0.2 mL of dried skimmilk extender (25 x 10^6 pms/mL) at 34 h (n=10) or 40 h (n=10) after GnRH administration. Mares receiving 1 mL were inseminated with a flexible plastic AI pipette, while mares receiving 0.2 mL were inseminated using a disposable implant gun (Veterinary Concepts, Green Valley, WI) containing a 0.5-mL plastic straw. Different insemination pipettes were used to optimize delivery of the 2 volumes. Semen was deposited at the tip of the uterine horn, ipsilateral to the preovulatory follicle. The location of pipettes within the uterus was confirmed by transrectal ultrasonography prior to semen deposition.

After insemination, mares were examined daily to determine the day of ovulation. Pregnancy examinations were performed by ultrasonography on Days 12, 14 and 16 postovulation (day of ovulation = Day 0).

Semen collection. Semen was collected with a Colorado model artificial vagina (Animal Reproduction Systems, Chino, CA) from 2 Arabian breed stallions of known high fertility immediately prior to the planned insemination. Ejaculates were evaluated for gel-free volume, sperm concentration and motility. Semen was then extended with a commercial skimmilk glucose extender (EZ-Mixin®) to either 25 x 10^6 prms/mL (n=51) or 5 x 10^6 prms/mL (n=10). Semen was kept at room temperature until inseminations were performed, within an hour after collection.

Experiment 2: Sexed Spermatozoa

Mares. Seventeen reproductively normal, cyclic mares of light-horse breeds, ranging in age from 5 to 12 yr, were used from August 15 to September 25, 1998, for a total of 21 cycles. Estrus was synchronized and ovulation was induced as described for Experiment 1. Four mares that failed to become pregnant from the first insemination were used a second time. Mares were randomly assigned to 1 of 2 groups.

Group A. Mares (n=11) were inseminated on a single occasion with ~25 x 10^6 live, sorted sperm cells in 1 mL (25 million/mL) 34 h after GnRH administration. Spermatozoa were sorted into a commercial skimmilk semen extender (EZ-Mixin®), centrifuged and resuspended in the same extender to a concentration of 25 x 10^6/mL. Sperm cells were deposited at the tip of the uterine horn, ipsilateral to the preovulatory follicle, using a flexible plastic AI pipette (I.M.V.). The location of the pipette within the uterus was confirmed by transrectal ultrasonography prior to semen deposition. One mare was inseminated with 20 x 10^6 live-sorted spermatozoa because of time constraints with the flow cytometer on that day. One mare failed to ovulate and was excluded from the study.

Group B. Mares (n=10) were inseminated as described for Group A except that 4% egg yolk was added to the extender. One mare was inseminated with 20 x 10^6 live-sorted spermatozoa because of time constraints with the flow cytometer on that day.

After insemination, mares were examined daily to determine the day of ovulation (Day 0). Pregnancy examinations were performed by ultrasonography on Days 12, 14, 16 and 30 post-ovulation, and fetuses were sexed between Days 60 and 70.

Semen collection and preparation. Two Arabian breed stallions of known high fertility were used in this experiment; one stallion had been used in Experiment 1. Semen was collected and evaluated as described for Experiment 1. Semen was extended 1:1 in HBGM-3 (24) and immediately transported at ambient temperature to the laboratory for further processing. It was centrifuged for 19 min at 400 x g at 22°C to concentrate the spermatozoa. After centrifugation, the supernatant was aspirated, leaving a soft 0.25-mL sperm pellet. The concentration of spermatozoa in the pellet was determined using a Densimeter® (Animal Reproduction Systems). The sperm cells were subsequently diluted to 400 x 10^6/mL in HBGM-3, then 25 µL Hoechst 33342 stock solution (5 mg/mL, water) were added to the 1-mL sperm suspension. Eight tubes (1 mL each) were prepared and incubated at 34°C for 1 h. Next, the stained samples were diluted to 100 x 10^6 sperm/mL by adding 3 mL of HBGM-3. Food coloring (2 µL/mL of 1%

FD&C #40 in HBGM-3) was added to each of the 8 sample tubes, resulting in a 4-mL total volume. Samples were then filtered through a 40-µ mesh filter apparatus into 6-mL polypropylene tubes and held at ambient temperature for up to 6 h until approximately 25 x 10^6 live sperm were sorted for DNA content by flow cytometry. Argon lasers, emitting 150 mW at 351 and 364 nm, were used on MoFlo® flow cytometer cell sorters modified for sperm sorting, operating at 50 psi, with HBGM-3 minus BSA as sheath fluid. Spermatozoa were collected at a rate of approximately 900 live cells/sec into 6 polypropylene tubes (14 mL each) containing 4 mL catch fluid, either EZ-Mixin® CST or 4% egg yolk in EZ-Mixin® CST, before the start of sorting. When 2 meters were available for insemination on the same day, both X and Y chromosome-enriched spermatozoa were collected. Tube contents were mixed every 30 min during sorting. After sorting, the spermatozoa were pooled from the 2 flow cytometers, placed in 50-mL centrifuge tubes, and centrifuged for 20 min at 1,200 x g at 22°C. The supernatant was then aspirated to a 200-µL sperm pellet, and 100 µL of post-centrifugation extender were added to the pellet; this was then transferred to a 50-mL plastic tube. A hemacytometer count was used to determine the final sperm concentration. Samples were then diluted to a total of 25 x 10^6 live, sorted sperm cells in a volume of 1 mL of the appropriate extender for use in AI.

Reanalysis of spermatozoa for DNA content. The relative DNA content of the sorted, live sperm cells used for insemination was determined by flow cytometry analysis of sperm nuclei from <8.5-mL samples of each batch collected at the end of the day. Sperm nuclei were prepared from an aliquot of intact sorted spermatozoa by sonication for 3 sec with an ultrasonic disintegrator 60 (Fisher Scientific) at #2 setting (approximately 1 watt). The proportion of X and Y chromosome-bearing spermatozoa was determined by fitting a pair of Gaussian distributions to the histograms from the O* detector (14). Reanalysis for DNA indicated an average sorting purity of 90% for X and 84% for Y chromosome-bearing spermatozoa for the 17 sortings.

Fetal sex determination. Fetuses from mares pregnant 60 to 70 d postovulation were sexed by transrectal ultrasonography without knowledge of the sex of the sorted spermatozoa at insemination. A real-time ultrasound scanner (Aloka 500®) equipped with a linear-array, 5-mHz transducer was used. Fetal sex can be accurately determined in horses and cattle by identifying and locating the genital tubercle (5).

RESULTS

Experiment 1

Pregnancy rates were not significantly different between stallions (Stallion A = 22/31, 71%; Stallion B = 15/30, 50%; P>0.1), or between mares at 34 vs 46 h after GnRH administration (19/29, 65% and 18/32, 56%, respectively; P>0.1), so the data were combined. Mares inseminated with 500 x 10^6 pms in a 20-mL volume had a significantly higher (P<0.05) pregnancy rate than mares inseminated with 25 or 5 x 10^6 pms (Table 1). There was no significant difference (P>0.1) in pregnancy rates between mares inseminated with 25 x 10^6 pms and those with 5 x 10^6 pms in a volume of 1 or 0.2 mL.

Although fertility was significantly higher in Group 1 with 500 x 10^6 pms than in Group 2 with 25 x 10^6 pms, a rate of 57% was achieved with a single insemination. This was not significantly different than pregnancy rates achieved with the 2 subsets in Group 3 (5 x 10^6 pms), 7/20 (35%), but this difference probably would be significant with larger numbers per treatment.

Table 1. Pregnancy rates from a single insemination

No. of progressively motile spermatozoa (x 10^6)	% Pregnant at Day 16
500 in 20 mL	18/20 (90%)[a]
25 in 1 mL	12/21 (57%)[b]
5 in 1 mL	3/10 (30%)[b]
5 in 0.2 mL	4/10 (40%)[b]

[a,b] Values with different superscripts differ (P<0.05, Chi square).

Experiment 2

Pregnancy rates at Day 16 postovulation after insemination of sex-sorted spermatozoa are shown in Table 2. Data from 1 mare ovulating 4 d postinsemination was not included in the results. Pregnancy rates were not different between stallions (Stallion A = 3/10, 30%; Stallion B = 5/10, 50%; P>0.1), so the data were combined. There was no difference in pregnancy rates between sperm treatments (EZ-Mixin = 3/10, 30%; 2Y + EZ-Mixin = 5/10, 50%; P>0.1). The phenotypic sex ratio of the 3 fetuses based on ultrasound examinations at 2 mo of gestation was predicted with perfect accuracy.

Table 2. Pregnancy rates following insemination with 25 x 10^6 sexed spermatozoa

Treatment group	No. of[a] mares inseminated	No. of mares pregnant at 16 days	No. of mares pregnant at 60 days	Predicted[b] sex (%)		Actual sex	
				♂	♀	♂	♀
EZ-Mixin	10	3[a]	1	Lost pregnancy[c]	89	Lost pregnancy[c]	1/1
4% 2Y+ EZ-Mixin	10	5[a]	4	84	87	3/3	1/1

[a] No significant difference (P>0.1; Fisher's Exact Test).

[b] Results of reanalysis for relative DNA content of aliquots of sorted X- and Y-bearing sperm populations.

[c] Lost pregnancy prior to sex determination.

Three mares lost their pregnancy prior to Day 60 postovulation, so fetal sex could not be determined. Two of these mares lost their pregnancies between Days 16 and 35, and the other mare between Days 35 and 50. One mare inseminated with X chromosome-bearing spermatozoa was euthanized at Day 66 of gestation due to a gastrointestinal problem. A phenotypically normal female fetus (the correct sex) was detected at necropsy.

The timing of insemination relative to GnRH administration was changed from 40 to 34 h post GnRH during Experiment 1 because many mares were ovulating prior to planned insemination and, therefore, were not inseminated. Data from 22 mare cycles (26.5%) were excluded because the mares either ovulated prior to planned insemination (n=11), did not ovulate (n=3), or ovulated >4 d after GnRH administration (n=8, Table 3). In Experiment 2, all mares were inseminated 34 h post GnRH administration, and no mares ovulated prior to the planned insemination (Table 3).

Table 3. Timing of ovulation relative to GnRH administration

Experiment I				Experiment II	
Insemination 40 hours post GnRH		Insemination 34 hours post GnRH		Insemination 34 hours post GnRH	
Time after GnRH (hours)	No. of mares ovulating	Time after GnRH (hours)	No. of mares ovulating	Time after GnRH (hours)	No. of mares ovulating
0[b]	0	0[b]	0	0[b]	0
12	0	24	0	24	0
36	10[a]	28	1[a]	28	0
40[c]	17	34[c]	1	34[c]	2
48	15	48	28	48	18
>96	4[a]	>96	2[a]	>96	1[a]
no ovulation	4[a]	No ovulation	1[a]	No ovulation	1[a]
Total	50	Total	33	Total	21

[a] Data excluded due to early ovulation, no ovulation or ovulation >4 d post GnRH administration.
[b] Time of GnRH administration.
[c] Planned time of insemination.

DISCUSSION

The number of spermatozoa commonly recommended per insemination dose for maximum fertility in mares is 500 x 10^6 pms every other day while the mare is in estrus. However, some studies have shown no decrease in fertility with inseminates as low as 100 x 10^6 pms (7,25). Studies using 50 x 10^6 motile spermatozoa have shown a decrease in fertility when compared with inseminates of 100 and 500 x 10^6 pms (26). Other studies have shown that as the number of inseminations increases due to longer estrus, fertility increases (35). However, the results from these studies have been inconsistent. Therefore, we examined in this study whether the use of low numbers of spermatozoa would provide reasonable pregnancy rates when administered on a single occasion, close to ovulation. This is of particular interest when inseminates consist of sexed spermatozoa, but also may be utilized on occasions when semen is of limited supply, such as from very popular stallions or old stallions, or when using frozen semen.

In Group 3 of Experiment 1, 20 mares were inseminated with 5 x 10^6 pms at a volume of 1 mL (5 x 10^6 pms/mL, n=10), or 0.2 mL (25 x 10^6 pms/mL, n=10). Pregnancy rates between the 2 subgroups were compared because they have been reported to decrease when diluting semen to <25 x 10^6 pms/mL (11). However, in this experiment there was no significant difference in fertility between the 2 sperm concentrations (1 mL, 5 x 10^6 pms/mL vs 0.2 mL, 25 x 10^6 pms/mL).

When inseminations were performed 40 h post GnRH administration, 10 of 50 mares (20%) ovulated prior to the planned insemination and thus were not inseminated at that cycle. Mares were palpated in the morning (between 8 and 10 am), and if a mare had a 35-mm follicle and was in estrus on that day, the GnRH implant was inserted at 8 pm. At implant insertion, follicular data were not recorded, so the actual size of the follicle was not known (follicles were ≥35 mm 12 h earlier). However, the preovulatory follicle was probably closer to 40 mm in size at the time of GnRH implant insertion. This explains why mares were ovulating earlier than expected (between 12 and 36 h post GnRH administration). We therefore decided to inseminate the remaining mares 34 h post GnRH administration. The mares were palpated in the morning, and if a mare was a candidate for GnRH, the implant was administered immediately (8 am) and the mare inseminated 34 h later. Only 1 mare ovulated prior to the planned insemination using this protocol.

If a mare had already ovulated based on palpation and ultrasound examination on the morning of the planned insemination, she was not inseminated. Instead, cloprostenol (250 µg) was administered 5 d postovulation to induce luteolysis so the mare could be reused. Pregnancies in Experiment 1 were terminated at Day 16 by locating the embryonic vesicle by transrectal ultrasonography and then disrupting it. Cloprostenol (250 µg, im) was then administered to induce luteolysis so mares could be reused.

Apparently this is the first report of inseminations in mares with 25 and 5 x 10^6 pms at the tip of the uterine horn. Pregnancy rates of 90% (18/20), 57% (12/21) and 35% (7/20) for Group 1 (500 x 10^6 pms), Group 2 (25 x 10^6 pms) and Group 3 (5 x 10^6 pms), respectively, were higher than expected, possibly due to: 1) use of stallions of known high fertility, and 3) actively

managing each mare's reproductive activity to allow inseminations to be performed within hours of ovulation. Data from 11 cycles (13%) of mares that did not ovulate or ovulated >96 h post GnRH administration were excluded because the objective of our study was to determine pregnancy rates when a single insemination was performed close to ovulation. Theoretically, if culling had not taken place, a Day 16 pregnancy rate of approximately 77% would have been achieved with a single 500 x 10^6 *post* insemination.

Semen was deposited at the tip of the uterine horn ipsilateral to the preovulatory follicle for the 2 lower dose inseminates in this study. It was thought that semenal deposition deep into the uterine horn would maximize fertility when using low numbers of spermatozoa in a low volume; however, this hypothesis was not tested in this study. Further studies are needed to evaluate the effect of site of sperm deposition on fertility.

Many attempts have been made during the past 80 yr to separate X and Y chromosome-bearing spermatozoa (33). The only reliable method of accurately identifying X and Y chromosome-bearing spermatozoa is flow cytometry/cell sorting based on DNA content (Beltsville sperm sexing technology). This technology makes it possible to alter the sex ratio with 90% accuracy (13), and the method has been used to obtain pregnancies following surgical insemination in rabbits (15, 22), swine (32) and horses (29). Surgical insemination was chosen in these earlier experiments because of the limited number of spermatozoa available due to the slow sorting rate (~100 sperm/sec) of X and Y sperm cells, and the apparent need for large numbers of spermatozoa to establish a pregnancy. Production of X- and Y-bearing spermatozoa per unit time by means of high speed sorting and a newly-developed nozzle have increased sorting rates 10 to 15 times that previously possible (17), thus enabling researchers to obtain pregnancies using laparoscopic insemination in sheep (4) and nonsurgical, intrauterine insemination in cattle (1, 30, 31, 32).

The present study was the first to obtain viable pregnancies in the horse following non-surgical, intrauterine insemination with sexed spermatozoa. The pregnancy rate at Day 16 following insemination of 75 x 10^6 sexed cells (40%) was not statistically different ($P>0.1$) from that of mares in Experiment 1 inseminated with 25 x 10^6 nonsorted, progressively motile cells (57%). The same insemination technique as well as the same mares and technicians were used in both experiments. Further, both experiments were conducted during the same breeding season, at approximately the same time of year. Because the pregnant mares in Experiment 1 were given prostaglandin at Day 16 and recycled, comparisons of pregnancy rates at Day 60 between the 2 experiments was not possible.

Three of eight mares (38%) inseminated with sexed sperm cells lost their pregnancies between 16 and 60 d. Two of these mares developed embryonic vesicles which appeared normal until day 16. The vesicles then decreased in size until they were no longer present. One of the three mares developed a viable pregnancy with a visible fetus with a heartbeat. The fetus was observed to be alive at Day 35, but was dead by Day 50. With fresh, nonsorted spermatozoa, early embryonic loss has been reported to be 9% by Day 14 and an average of 16% between Days 20 and 50 (34). In cattle, Seidel et al. (32) reported only 1 pregnancy loss from 34 pregnant heifers between Days 30 and 33 and Days 64 and 67 in groups inseminated with sexed

spermatozoa and frozen-thawed control semen, respectively. The same sperm staining and sorting procedure used in the above study was used in our present study. It is possible that equine spermatozoa are more sensitive to the staining and sorting procedures than bovine spermatozoa. Greater numbers of pregnancies from sexed spermatozoa are needed to determine if equine embryonic death is increased significantly with use of spermatozoa sorted by flow cytometry. Four mares pregnant with sexed spermatozoa in Experiment 1 produced live, healthy foals during the summer of 1999.

The slightly lower pregnancy rates resulting from sexed spermatozoa are probably due to the time required to sort 25 x 10^6 cells, and/or possible damage to the spermatozoa by the sorting process. In this study, the average time from semen collection to insemination was 7 h. In the first experiment, mares were inseminated almost immediately after semen collection. The average total and progressive motility for the sexed spermatozoa was 69 and 38%, respectively, and a total of 25 x 10^6 live-sorted sperm cells was collected for insemination. The sorting process is stressful for spermatozoa since they are pumped through fine tubing at a high pressure, causing them to exit at ~100 km/h; stored diluted at 600 x 10^6 sperm/mL; and held at ambient temperature for several hours until adequate numbers are collected. Before sorting, the spermatozoa were incubated for 1 h at 35°C with Hoechst 33342, which has a high affinity for AT-rich regions of DNA, and then exposed to laser light at 351 and 364 nm. Ultraviolet light in a broader spectrum of wavelengths causes an increase in chromosomal structural abnormalities in mouse spermatozoa (20). The safety of Hoechst 33342 has also been debated (16, 23). Unlike many DNA-specific stains, Hoechst 33342 does not intercalate into the DNA helix; however, this does not prove that the stain is not toxic. None of these processes is conducive to sperm viability, although it is not clear which if any of these aspects of the process cause the most damage. To date, no increased incidence of genetic abnormalities has been reported in the hundreds of offspring of various species that have been produced utilizing this technology.

McNutt and Johnson (22) proposed another possible explanation for lower pregnancy rates with sexed spermatozoa. They found that the first cell cycle was delayed in rabbit embryos fertilized by sperm cells treated with Hoechst 33342. However, the mechanism by which this occurs is not known but could be due to interference of the dye molecules as the DNA is replicated or transcribed. Decreased embryo survival also has been documented in flow-sorted spermatozoa (3, 15).

In summary, a Day 16 pregnancy rate of 57% was achieved with a single insemination, close to ovulation, with 25 x 10^6 sperm when deposited deep into the uterine horn. We have demonstrated for the first time that pregnancy in the mare can be achieved, and foals of predetermined sex can be obtained, by deposition of a low number of spermatozoa at the tip of the uterine horn of the mare. Further experiments are necessary to determine if there is a less invasive way to treat equine spermatozoa and still obtain high numbers of sexed cells per unit of time. Sexing mammalian spermatozoa remains a research technique, but it could be available to commercial AI programs in the near future.

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Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions

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Abstract

The present study was performed to test fertility after low dose insemination with sexed and non-sexed sperm in dairy cattle under field conditions in Switzerland. Spermatozoa were stained with Hoechst 33342 and sorted by flow cytometry. A total of 132 heifers and cows were inseminated with 2×10^6 X-bearing, frozen-thawed sperm (A) and 91 animals were inseminated with the same dose using non-sorted, frozen-thawed sperm (B). Pregnancy examination by ultrasound was performed twice, 30–40 days (PE1) and 70–90 days (PE2) after insemination. The pregnancy rates after PE1 were 33.3% (9/27) and 59.3% (16/27) in heifers ($P = 0.05$) and 27.6% (29/105) and 28.1% (18/64) in cows ($P > 0.05$) for groups A and B, respectively. Embryonic losses between PE1 and PE2 in heifers were 11.1% (1/9) and 0% (0/16) and in cows 17.2% (5/29) and 5.6% (1/18), the differences between groups A and B not being significant ($P > 0.05$). Calving rates in heifers were 29.6% (8/27) and 57.8% (15/26), whereas in cows 22.1% (23/104) and 23.4% (16/63) gave birth to calves (for both groups $P > 0.05$). The sex ratio was different ($P < 0.05$) between A (85.3%) and B (58.6%). From our results it can be concluded that conception rates of sorted and non-sorted semen are similar using an insemination dose of 2×10^6. Fertility may be increased by improving sexing technology and animal management.

Keywords: Sexed semen; Low dose insemination; Cows; Heifers; Fertility

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1. Introduction

Until now, the only reliable method for separating X- and Y-chromosome-bearing spermatozoa is flow cytometrical cell sorting for DNA content of sperm. The first live offspring from flow cytometrically sorted sperm resulted from rabbits surgically inseminated into the oviduct [1]. From then on, offspring of pre-selected sex have been produced with an accuracy close to 90% in pigs [2–4], cattle [5–8], horses [9,10] and humans [11]. Due to improvement of nozzle design [12] and adaptation to high-speed cell sorting [13] the efficiency of the sexing procedure has increased considerably. Accuracy of the desired sex ranges from 85 to 90% and sorting speed has been established at up to 11×10^6 sperm cells/h [13]. The sexing method has also been combined with in vitro fertilization in pigs [2,3] and cattle [14,15]. To make sorted sperm available for artificial insemination on a commercial basis, experiments using low dose insemination with flow-sorted sperm were performed in horses [9,10], cattle [6,7], sheep [16] and pigs [4,17]. In addition, it is known that during sorting with the flow cytometer, dead sperm cells are not sorted, and therefore not loaded into straws [18].

To increase fertility, uterine horn insemination with sorted frozen-thawed sperm was tested in cattle [6]. Results of these experiments did not show a significant increase of pregnancy rates compared with uterine body insemination, although some authors [19] claim to have better results when performing uterine horn insemination with non-sorted sperm. Seidel et al. [6] combined sorted, liquid semen with very low dose ($(1–2) \times 10^5$ in 0.1 mL) insemination into the uterine horn ipsilateral and contralateral to the ovary bearing the largest follicle. Pregnancy rates for ipsilateral and contralateral inseminations were nearly identical and ranged from 2.6 to 22.4%, depending on the time between the end of sorting and insemination as well as on the bull used. A recent study in pigs [4] shows, that low dose (70×10^6 spermatozoa) insemination with flow cytometrically sorted sperm deep into the uterine horn resulted in pregnancy rates of 45.6 and 35% for induced and spontaneous ovulation, respectively. In horses, hysteroscopic insemination into the uterine horn [10,20] and ultrasound guided deep uterine insemination [9,20] were performed using sex-sorted sperm in low concentrations (5×10^6 sperm cells/dose). Hysteroscopic insemination resulted in more pregnancies than ultrasound guided deep uterine insemination [10,20].

Reduced fertility, when using sorted sperm, has been attributed to damage of spermatozoa caused by the sexing process [18]. This includes staining and incubation of spermatozoa with Hoechst 33342, sperm dilution, exposure to high pressure and laser light, the rapid projection into the collection tube and also centrifugation to concentrate sorted sperm. After sorting, spermatozoa are partially capacitated resulting in a shorter life span and consequently in reduced fertilizing capacity [4]. To increase sperm quality and fertility of sexed sperm, Seidel et al. [21] as well as Suh and Schenk [22] showed that post-thaw motility and fertility were considerably higher when lower pressure was used during sorting. This however, reduces the sorting rate by 2–3% [22]. Embryo survival rates were also impaired with sorted sperm in cattle [6], pigs [4], mares [9] and rabbits [23] when compared with non-sorted sperm but calves produced with sexed sperm were not different from control calves regarding birth weight, mortality and weaning weight [24,25]. Furthermore no differences were observed between cows carrying calves derived from

sorted or non-sorted sperm when comparing gestation length, abortion rate and calving ease [25].

Since little information exists about low dose insemination with sexed sperm in Switzerland, the objective of the present study was to evaluate fertility in heifers and cows inseminated with low numbers of sexed and non-sexed frozen-thawed sperm under field conditions.

2. Material and methods

2.1. Semen collection and quality control

For this study, semen of seven bulls of different ages and breeds (five Brown Swiss, two Red Holstein) was collected using an artificial vagina. After collection, sperm concentration was determined with a spectrophotometer (Co75 Colorimeter, WPA, Cambridge, UK). Mass motility and progressive sperm motility were evaluated subjectively by phase contrast microscopy (CX 40 Olympus, Volketswil, Switzerland). Only ejaculates with a concentration of more than 800×10^6 spermatozoa/mL and a progressive motility of at least 80% were further processed. Thereafter, ejaculates were divided in half to produce sex-sorted sperm and non-sorted sperm. The percentage of ejaculates from all seven bulls fulfilling the above criteria varied between 25 and 64%.

2.2. Flow cytometric sorting of sperm

Sperm sorting was performed with a MoFlo® SX (Cytomation Inc., Fort Collins, USA) equipped with an argon laser, detectors for fluorescence and a special spermatozoa oriented nozzle developed by Rens et al. [12]. An aliquot of freshly collected semen was diluted in staining medium (modified Tyrode's albumin lactate pyruvate (TALP)) to a concentration of 200×10^6 spermatozoa/mL followed by staining with Hoechst 33342 for 60 min at 37 °C. The sample was diluted in the sorting medium (TALP containing 20% egg yolk) and vital-staining food dye (Sigma–Aldrich, Fluka Chemie GmbH, Buchs, Switzerland) was added to mark sperm cells with ruptured membranes [18]. During sorting at room temperature (90 min), spermatozoa in the sorting medium were mixed with a Tris-based sheath fluid containing citric acid and fructose. Sperm cells showing fluorescence when excited by the argon laser passed two detectors at 90° to each other, one to diagnose correct orientation and the other to measure strength of fluorescence. According to the amount of emitted fluorescent light, the spermatozoa were given an electric charge, i.e. X-bearing sperm were negatively charged and attracted to the positive field on the right, while Y-bearing sperm as well as badly oriented spermatozoa and cells with ruptured membranes containing food coloring, were not electrically charged and ended in a waste tube [18].

Sorted spermatozoa were collected into tubes containing catch fluid (22% egg yolk–Tris-extender), allowed to equilibrate at 5 °C for a minimum of 90 min and centrifuged at $85 \times g$ for 20 min. After resuspension with Tris-based extender, concentration was assessed using an improved Neubauer hemocytometer and brought to the final concentration of 8×10^6 spermatozoa/mL by adding Tris-based extender containing

glycerol. The sample, with a final concentration of 6% glycerol, was kept in a cooling cabinet at 5 °C until packaging. The non-sorted portion of an ejaculate was diluted to a concentration of 16×10^6 spermatozoa/mL with Tris-based extender and then cooled down to 5 °C. Finally the non-sorted ejaculate fraction was diluted to a concentration of 8×10^6 spermatozoa/mL with Tris-based extender containing glycerol.

2.3. Further processing and freezing

Sorted and non-sorted sperm were packaged at the same time in 0.25 mL French straws (IMV, Aigle, France), each containing 2×10^6 spermatozoa. After packaging, straws were kept on freezing racks at 5 °C for 3–4 h and then frozen in an automatic freezer (No. 5109, Nifa Instrumenten BV, Leeuwarden, The Netherlands) and stored in liquid nitrogen at −196 °C. After freezing, one straw of each batch was thawed at 38 °C for 20 s and quality parameters were established. Sexed semen with less than 15% progressive motility and control doses with less than 30% progressive motility were discarded. In addition, one straw of each sorted batch was sonicated and analyzed by flow cytometry to determine accuracy of sorting which was required to be greater than 85%.

2.4. Artificial insemination

Artificial insemination (AI) was performed by professional personnel, including one technician and five veterinarians. Animals were inseminated 12 h after the occurrence of standing heat and were not synchronized. Inseminators were blind to whether they were using sorted or non-sorted straws. Straws were thawed at 38 °C for 20 s and inseminations were performed into the uterine body.

2.5. Field study

For this study, a total of 27 heifers and 105 cows (up to lactation number 4) were inseminated with sex-sorted, frozen-thawed sperm (group A) and 27 heifers and 64 cows (up to lactation number 4) with non-sorted, frozen-thawed sperm (group B). Cows were between 40 and 90 days post partum. All straws used in this trial contained 2×10^6 spermatozoa.

Heifers and cows of the Brown Swiss and the Red Holstein breeds were scattered on 117 farms in different regions of Switzerland. Because of heterogeneous farm structures with very low numbers of animals per farm, parameters such as milk yield, body condition and feeding program were not recorded in this study. Problem animals, defined as females with impaired function of the genital tract, severe foot problems or systemic illness were not inseminated. Since inseminations have been performed from July 2002 to February 2003 seasonal influences could not be excluded. All animals not returning to estrus 21 days after insemination were examined by ultrasound (Tringa, Esoate Pie Medical, Maastricht, The Netherlands) for pregnancy 30–40 days after insemination (PE1). Pregnant animals were re-examined sonographically between 70 and 90 days after insemination (PE2). Failure to confirm pregnancy at PE2 represented embryonic loss. Animals returning to service or not being pregnant when examined

30–40 days after insemination were not further evaluated. Calving ease, possible dystocias and sex of the newborn calves were recorded by information provided by the farmers.

2.6. Statistical analysis

Statistic analysis was performed with Stat View 5.0[®] (SAS Institute, Wangen, Switzerland). A χ^2-test was performed to compare rates of pregnancy, abortion and calving between treatments. In addition, a logistic regression model was used to analyze the effects of animals, bull, inseminator and treatment on pregnancy, abortion and calving. Differences were considered significant at $P < 0.05$.

3. Results

When analyzing fertility data of all animals including heifers and cows ($n = 223$), no significant difference ($P > 0.05$) could be observed between groups A and B. The conception rate 30–40 days after insemination (PR1) was 28.8% using sex-sorted sperm (group A) and 27.4% for non-sorted sperm (group B). At 70–90 days after insemination pregnancy rates (PR2) were 24.2 and 36.3% for sexed and non-sexed sperm, respectively ($P > 0.05$). Embryonic loss was calculated to be 15.8% for group A and 2.9% for group B ($P > 0.05$) and the calving rate was 23.5 and 33.0% for group A and B, respectively ($P > 0.05$). Given the fact that fertility in heifers is generally higher than in cows, data presented in Table 1 were separately analyzed for heifers and cows.

In heifers, pregnancy rates at 30–40 days (PR1) and 70–90 days (PR2) after insemination were significant ($P = 0.05$) between groups A (33.3%, 9/27) and B (59.3%, 16/27). Comparing pregnancy results between heifers and cows a clear difference could only be detected in group B ($P = 0.005$). In group A, one heifer out of nine (11.1%) lost its embryo between PE1 and PE2 compared to none out of 16 in group B ($P > 0.05$). Calving

Table 1
Pregnancy rates (PR), abortion rates (AR) and calving rates (CR) in heifers and cows inseminated with sex-sorted (A) and non-sorted (B) semen

Group	Animals	Pregnant at (PE1)	PR1 (%)	Pregnant at (PE2)	PR2 (%)	Embryonic loss	AR (%)	Animals calving	CR (%)
A	132	38	28.8	32	24.2	6	15.8	31	23.7
B	91	34	37.4	33	36.3	1	2.9	31	34.8
Heifers A	27	9	33.3 a	8[a]	29.6 a	1	11.1	8	29.6
Heifers B	27	16	59.3 b	16	59.3 b	0	0.0	15[b]	57.8
Cows A	105	29	27.6 a	24[c]	23.8 a	5	17.2	23[c]	22.1
Cows B	64	18	28.1 c	17[d]	26.6 c	1	5.6	16[d]	23.4

PR1: pregnancy rate 30–40 days after insemination, PR2: pregnancy rate 70–90 days after insemination. Different letters within a column indicate statistically significant differences ($P < 0.05$).

[a] One heifer lost its embryo between PE1 and PE2.

[b] One heifer died before calving.

[c] Five cows lost their embryos between PE1 and PE2 and one animal was sold before calving.

[d] One cow lost its embryo between PE1 and PE2 and another was slaughtered before calving.

rate in heifers was not significantly different ($P = 0.098$) between group A (29.6%, 8/27) and B (57.8%, 15/26).

In cows, pregnancy rates after PE1 did not differ ($P > 0.05$) between group A (29/105, 27.6%) and B (18/64, 28.1%), respectively and also for PR2 the difference between group A (23.8%, 24/105) and B (26.6%, 17/64) was not significant ($P > 0.05$). The percentage of embryonic loss in cows between PE1 and PE2 was 17.2% (5/29) in group A and 5.6% (1/18) in group B ($P = 0.40$) and calving rates were 22.1% (23/104) and 23.4% (15/64) for groups A and B, respectively ($P > 0.05$).

With the exception of one bull with a significant ($P = 0.04$) effect on pregnancy rates of heifers in group B, the effects of season, breed, number of lactation (tested only in cows) and inseminator on pregnancy rates, embryonic loss and calving rate were not significant ($P > 0.05$). When comparing the calving rate with the PR2 in heifers as well as in cows, no significant difference could be detected ($P > 0.05$). When determining the sex of the newborn calves more female calves ($P < 0.05$) were born in group A (29/34, 85.3%) than in group B (17/28, 58.6%) and more twins ($P < 0.05$) were born in group A than in group B. Between PE2 and term no fetal malformation or abortions occurred in heifers and cows.

4. Discussion

Compared to Seidel et al. [7] our pregnancy rates obtained from inseminations with a dose of 2×10^6 spermatozoa into the uterine body were low, irrespective whether sex- or non-sorted sperm was used. In cows, pregnancy rates were lower than 30% and did not differ between groups A (sorted) and B (non-sorted) indicating that the total number of spermatozoa inseminated seems to be more detrimental for conception than the use of sexed or non-sexed sperm. This also agrees with findings of Andersson et al. [26], who reported significantly lower conception rates using 2×10^6 spermatozoa/dose compared to 15×10^6 spermatozoa/dose. We have to take into account, that the sorted cells are not only sorted for sex, but are also sorted for membrane integrity, thus only vital cells are sorted.

In heifers, using the same insemination dose as in cows, conception rate with sex-sorted sperm was not significantly different from cows (33.3% versus 27.6%), but was clearly increased when non-sorted sperm was used (59.3% versus 28.1%). In the US, Seidel et al. [7] reported conception rates in heifers around 50% when using sex-sorted sperm at doses between 0.3 and 3.0×10^6. These results might be compared to data obtained by another study (unpublished data) of Big-X, Switzerland, including heifers (47%) and lactating cows (32%) with an insemination dose of 2×10^6 sexed sperm. A possible cause for the low pregnancy rates in heifers with sex-sorted sperm in the present experiment may be the combination of the low insemination dose together with low post-thaw motility, the fragility of sorted spermatozoa and the heterogeneous management conditions including estrus detection, timing of AI as well as the feeding and animal health programs. This agrees with the work of Seidel [8] who also found low conception rates if management conditions were not optimal. In contrast, pregnancy rates in heifers using non-sorted sperm were nearly 60% corresponding with calculated results of Den Daas et al. [27] when using the same insemination dose of 2×10^6. In addition, these results were found to strongly depend on the bull used for insemination. Therefore, caution must be applied when

interpreting our results with non-sorted sperm in heifers as one bull clearly influenced pregnancy rates of heifers in group B. The strong influence of individual bull fertility has also been confirmed by Seidel et al. [6].

Regarding embryonic loss between PE1 and PE2, the percentages in cows and heifers were higher when using sex-sorted sperm (17.2% versus 11.1%), than non-sorted sperm (0% versus 5.6%). These values, however, are lower when comparing to findings of Seidel et al. [6] who reported 23% of abortions between 1 and 2 months of gestation using sexed liquid sperm in heifers. The increased abortion rate during the first 2–3 months of gestation may be caused by treating sperm with DNA-binding fluorescent stain and damaging spermatozoa during flow cytometry. This hypothesis, however, has to be confirmed in future studies using a larger number of animals. Interestingly enough, abortions after three months of gestation or malformations in newly born calves were not observed in our study as well as in other studies [6,24,25] and a recent study including large numbers of animals inseminated with sorted semen reported abortion rates between 2 months of gestation and term of 4 and 2.4% for female and male calves, respectively [25].

According to Seidel et al. [7] higher conception rates were obtained when inseminating 3×10^6 spermatozoa compared to 1.5×10^6 spermatozoa/dose under heterogeneous field conditions. Den Daas et al. [27] calculated that for most bulls, the limiting sperm concentration to obtain around 80% of the normal conception rate is around 2×10^6 sperm cells/dose. Whether this is also applicable on flow cytometrically sorted sperm needs to be determined. In pigs [4] pregnancy rates of 45.6 and 54.3% were recorded with 70×10^6 and 140×10^6 sexed spermatozoa in combination with deep uterine insemination. In horses [20], the limiting sperm concentration is considered to be around 5×10^6 spermatozoa/dose, if either hysteroscopic or deep uterine insemination techniques are applied. Comparing data across species, it seems that in the bull a dose of $(2–3) \times 10^6$ spermatozoa is too low to obtain pregnancy rates around 80% of the normal.

As expected, the percentage of female calves born in group A was significantly ($P < 0.05$) higher than in group B which clearly demonstrates that flow-cytometric sorting of semen is a reliable method for sex pre-selection in the bovine.

From our field study, we can draw the conclusion that conception rates are similarly low when using sorted and non-sorted semen at doses of 2×10^6 spermatozoa. We therefore suggest that fertility may be increased by selection of sires, improving the management of the female as well as optimizing the sorting process, cryopreservation and the insemination technique. For correct insemination time intense estrus control without or with facilitating devices, such as electronic mounting detectors [28] is required. This seems to be very crucial, especially when using sex-sorted spermatozoa, which are known to be partially capacitated before insemination and thus may negatively affect fertilization success especially when insemination time was inappropriate. Ranking et al. [29] investigated the periovulatory events in dairy heifers over three years and found that the time interval between onset of estrus and ovulation had a significant effect on conception rate. Walker et al. [30], using an electronic mounting detection system reported that ovulation takes place within 27 h after observation of first mount. To facilitate optimal insemination time, estrus synchronizing protocols should be tested in combination with sex-sorted sperm [31]. Another important point influencing semen quality and conception results is the system pressure during flow-sorting, which has not yet been adjusted to the improved and lower

level of 30 psi [22]. Following the sorting procedure, an optimal cooling rate at freezing [32] and careful handling of frozen semen by inseminators [33] are also necessary. Improvement of deep uterine insemination techniques with modern flexible catheters to bring spermatozoa closer to the functional sperm reservoir in the caudal portion of the Fallopian tube should be the goal of future experiments [34]. This, however, requires also the necessity for adequate training of veterinarians and AI technicians.

In summary, from our data it can be concluded that conception rates between sorted and non-sorted semen inseminated at doses of 2×10^6 are similar. Fertility may be increased by improving several factors such as careful selection of AI bulls, accurate estrus detection and time of insemination as well as optimized semen processing, handling and insemination technique.

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Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen–thawed sorted X- or Y-chromosome-bearing spermatozoa

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Abstract. The fertilizing ability of sex-sorted frozen–thawed ram spermatozoa was assessed after insemination of mature Merino ewes at a synchronized oestrus. Ewes were inseminated into the uterus or utero–tubal junction (UTJ) with a total of 140×10^6 unsorted (control) or $2–4 \times 10^6$ sorted (X or Y) frozen–thawed ram spermatozoa 54 to 57 hours after removal of progestagen-impregnated pessaries and an injection of 400 IU of pregnant mare serum gonadotrophin (Folligon®, Intervet). The spermatozoa were separated into X- and Y-chromosome-bearing spermatozoa after analysis with a modified high-speed cell sorter (SX MoFlo®). The number of ewes pregnant after insemination with unsorted frozen–thawed spermatozoa was significantly higher (26/48; 54.3%) than for ewes inseminated with either X- (12/48; 25.0%) or Y-sorted spermatozoa (7/48; 14.6%) ($P<0.05$). Seventeen of the eighteen lambs produced by ewes inseminated with X-sorted spermatozoa were female (94.4%) and 8/8 lambs from ewes inseminated with Y-sorted spermatozoa were male (100%). The sex ratio of the lambs born to ewes inseminated with sex-sorted spermatozoa was significantly skewed from the 51.3% male and 48.7% female ratio in the control group ($P<0.05$). This study showed, for the first time, that lambs of predicted sex can be produced after insemination with low numbers of sex-sorted cryopreserved ram spermatozoa.

Introduction

The predetermination of the sex of offspring using spermatozoa separated by fluorescence differential sorting has been achieved in several species (Johnson 2000). Successful cryopreservation of sorted spermatozoa and subsequent production of offspring after artificial insemination has only been reported in cattle (Seidel *et al.* 1999) and horses (Lindsey *et al.* 2002)

The commercial application of sex predetermination using sorted sperm has been made possible in cattle following the development of methods for successfully freezing and thawing the sorted spermatozoa (Schenk *et al.* 1999). Similar applications in the sheep industry are yet to take place, since only a few lambs have been produced from fresh (unfrozen) sorted spermatozoa (Catt *et al.* 1996; Cran *et al.* 1997).

The aim of the present study was to examine the fertilizing ability of sorted frozen–thawed spermatozoa after the insemination of low numbers into either the uterus or utero–tubal junction (UTJ) of ewes at a synchronized oestrus.

Materials and methods

Animals and experimental design

One hundred and forty-four mature Merino ewes were randomly allocated into three treatment groups. Inseminations were carried out early in the breeding season, in February 2001, at a commercial sheep farm located in the southern highlands of New South Wales, Australia. Ewes were maintained on pasture but were also provided with supplementary feed in the form of lupin grains and hay just prior to insemination and for 4 weeks before and during lambing. The time of oestrus and ovulation was controlled by progestagen-impregnated intra-vaginal pessaries (40 mg Chronogest®, Intervet, Bendigo, Victoria) inserted for 12 days. At sponge withdrawal, all ewes received a single intramuscular injection of 400 IU of pregnant mare serum gonadotrophin (PMSG, Folligon®, Intervet) to enhance their ovulatory response.

Preparation of spermatozoa for sorting

Semen was collected by artificial vagina from two Merino rams (Ram 1, Ram 2) during January and February 2001. The concentration of spermatozoa was determined by haemocytometer and motility was determined by subjective assessment (Evans and Maxwell 1987). Only ejaculates containing spermatozoa with forward progressive motility greater than 80% were used. Spermatozoa were diluted to a concentration of 400×10^6 mL^{-1} with a modified TALP medium (XY TALP; Schenk *et al.* 1999). Each 2 mL sample was incubated with either 355.9 μM (Ram 1) or 311.4 μM (Ram 2) of Hoechst 33342 (H33342; Sigma, St Louis, MO, USA) for 1 h at 34°C. The concentration of dye for each ram was determined by a series of optimization experiments. After 1 h incubation, stained samples were diluted slowly with 2 mL of filtered XY TALP containing 4% egg yolk (v/v) and 0.002% food dye (FD&C #40,Warner Jenkinson Company Inc., St Louis, MO, USA) to 200×10^6 spermatozoa mL^{-1}. The food dye quenches the intensity of fluorescence of the dead sperm in the sample by penetrating their membranes. Therefore only viable sperm are selected for sorting (Johnson *et al.* 1999). Immediately before sorting, samples were

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filtered through a 40-μm nylon mesh filter to remove any agglutinated spermatozoa or debris.

Flow cytometric sorting

A high-speed cell sorter (SX MoFlo®, Cytomation Inc., Fort Collins, CO, USA) modified for sperm sorting (Johnson and Pinkel 1986; Rens *et al.* 1999), operating at 50 psi with a pre-warmed Tris-based sheath fluid (Schenk *et al.* 1999), was used to analyse and separate the spermatozoa. The fluorescent dye was excited by an argon laser running at 350 mW. Gates were set during sorting so that purities of greater than 90% X- or Y-chromosome-bearing spermatozoa were achieved. Average flow rates during the experiment, which varied according to ram and ejaculate, were 20000–25000 events s^{-1} and the average sorting rates (which also varied with ram and ejaculate) for each of X- and Y-chromosome-bearing spermatozoa were 4000–5000 spermatozoa s^{-1}. Putative male and female spermatozoa were collected simultaneously into 10-mL centrifuge tubes, pre-soaked with 1% BSA in TRIS sheath fluid, containing 1 mL of warm, filtered XY TALP and 20% egg yolk (v/v). Each sample was sorted for 30–60 min (depending on the flow and sorting rates) to achieve a total yield of 16 million (8 million of each sex) spermatozoa in 8 mL of sheath fluid (2.5% egg yolk, v/v).

Reanalysis of sorted spermatozoa

At the completion of each 30–60-min sort, 200000 X- and Y-chromosome-bearing spermatozoa were collected, processed and analysed as described by Welch and Johnson (1999) to provide an estimate of the purity of each sort and a prediction of the proportion of male and female lamb offspring.

Freezing, thawing and assessment of sorted spermatozoa

Sorted spermatozoa were centrifuged at 700*g* for 6 min at 30°C and the 80-μL pellet was resuspended 1 : 4 (sperm pellet : diluent, v/v) in either (*i*) TEST buffer containing 20% egg yolk (v/v) (Johnson *et al.* 1989) and 3% glycerol (TBY; v/v) or (*ii*) zwitterion-buffered diluent containing 13.5% egg yolk and 6% glycerol (ZWIT; Molinia *et al.* 1996). Separate ejaculates were collected from each ram as control samples and were diluted 1:4 with TBY or ZWIT. Both sorted (X-sorted, Y-sorted) and unsorted (control) samples were cooled to 4–5°C in 1.5 hours and then frozen as 0.2-mL pellets on dry ice (Evans and Maxwell 1987) before being transferred to liquid nitrogen for storage until use. Two pellets prepared from the same ejaculate were thawed in a dry test tube shaken in a water bath at 37°C and used for insemination within 10 min of thawing. Motility was immediately assessed and recorded after thawing, and slides were made for the assessment of acrosome integrity by fluorescent isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining as described by Cross *et al.* (1986).

Artificial insemination

Inseminations were carried out 54–57 h after withdrawal of progestagen sponges. All ewes were fasted for 12 h before insemination and local anaesthetic (2% Lignocaine, Troy Laboratories, Smithfield, New South Wales, Australia) was injected at the site of abdominal puncture. Intrauterine inseminations were conducted by laparoscopy in 81 ewes as described by Evans and Maxwell (1987) and 0.1 mL of thawed semen was deposited into the lumen of each uterine horn. Utero–tubal junction inseminations were carried out in 63 ewes using a disposable, 3.5 french, 11.4 cm tom cat catheter (Sovereign®, Sherwood Medical, St Louis; MO, USA). After mini-laparotomy, a small puncture was made in the tip of the uterus, and the catheter was passed up to the UTJ, where 0.1 mL of thawed semen was deposited into each UTJ. A total of 140×10^6 spermatozoa per ewe, a commercial artificial insemination dose, was inseminated into 48 ewes in the control group (27 intrauterine and 21 UTJ) and between 2 and 4×10^6 total sorted spermatozoa were inseminated into each of the 48 ewes in the X- (26 intrauterine and 22 UTJ) and Y- (28 intrauterine and 20 UTJ) sorted groups.

Pregnancy diagnosis

Jugular blood samples were collected from each ewe on Day 18 after insemination and progesterone concentrations in blood plasma were determined with a commercially available radioimmunoassay kit (Spectra; Orion Diagnostics, Helsinki, Finland). Ewes with progesterone concentrations greater than 1.2 ng mL^{-1} were considered pregnant (Robertson and Sarda 1971). All ewes were scanned by ultrasound for detection of fetuses on Day 60. Pregnancy loss in ewes was calculated as (the number of ewes pregnant at Day 18 - the number of ewes pregnant at Day 60)/the number of ewes pregnant at Day 18.

Lambing

After Day 60, pregnant ewes were placed in sheltered paddocks and put on an increasing plane of nutrition leading up to and during lambing. One week before the commencement of lambing, each ewe inseminated with sex-sorted spermatozoa was housed in a separate pen in the shearing shed and was observed 24 h a day until the end of lambing. Every lamb born was weighed with a set of calibrated hand scales, identified for sex and ear tagged within 6 h of birth.

Statistical analysis

Data on pregnancy loss and pregnancy rate in ewes and the effect of ram and insemination site were analysed by logistic regression using the GENSTAT computer program (Version 4.2) (Numerical Algorithms Group[©] (NAG) Ltd, Oxford, UK). Lamb weight, post-thaw motility and acrosome status were analysed by ANOVA and logistic regression using GENSTAT. Proportional data for the sex ratio and re-sort analysis values were analysed by the chi-square test and the two-sample *t*-test respectively.

Results

Post-thaw motility and acrosome integrity of spermatozoa

There was no significant difference in the post-thaw motility of control (unsorted), X-sorted and Y-sorted samples. The percentage of motile spermatozoa (± SEM) after thawing was 46.2 ± 0.8%, 46.3 ± 1.0% and 42.3 ± 0.9% for control, X-sorted and Y-sorted samples respectively. Freezing diluent and ram also had no effect on the post-thaw motility or acrosome integrity of spermatozoa.

Sorted frozen–thawed spermatozoa had significantly more intact acrosomes after thawing than unsorted frozen–thawed (control) spermatozoa ($P<0.001$). The incidence of intact acrosomes after thawing was 80.6 ± 1.0%, 92.9 ± 0.5% and 91.1 ± 0.6% for control, X-sorted and Y-sorted samples respectively. Sorted samples from Ram 2 had significantly less spermatozoa with intact acrosomes (86.1 ± 0.9%) after thawing than Ram 1 (90.1 ± 0.79%) ($P<0.001$).

Pregnancy rate

Data on pregnancy at Days 18 and 60 are presented in Table 1.

Ewes inseminated with 140×10^6 unsorted frozen–thawed spermatozoa (control) had a higher pregnancy rate at Day 60 (54.2%) than ewes inseminated with either $2–4 \times 10^6$ X- (25.0%) or Y-sorted spermatozoa (14.6%) ($P<0.05$).

Table 1. Pregnancy loss between Day 18 and Day 60 after artificial insemination of ewes with control and sex-sorted frozen–thawed spermatozoa

Type of semen	No. ewes inseminated	No. pregnant at Day 18 (%)[A]	No. pregnant at Day 60 and lambing (%)[B]	Pregnancy loss[C]
Control	48	34 (70.8)[a]	26 (54.2)[a]	23.5
X	48	21 (43.8)[b]	12 (25.0)[b]	42.9
Y	48	15 (31.3)[b]	7 (14.6)[b]	53.3

[A]Determined by progesterone assay.
[B]Determined by ultrasound.
[C](No. ewes pregnant at Day 18 – no. ewes pregnant at Day 60)/No. ewes pregnant at Day 18) × 100.
Within columns, values with different superscripts differ significantly ($P<0.05$).

There was no significant difference in pregnancy rate at Day 60 between ewes inseminated with either X- or Y-sorted spermatozoa.

The site of insemination and freezing diluent had no significant effect on the proportion of ewes pregnant and there were no significant interactions between the treatments. The pregnancy rate for Ram 2 (17/72) was lower than for Ram 1 (28/72), though overall there was no statistically significant difference after analysis by logistic regression.

Pregnancy loss

The overall pregnancy loss estimated from Day 18 to Day 60 after insemination for the flock was 38.6 ± 9.60%. There was no difference in pregnancy loss for ewes inseminated with unsorted control spermatozoa compared with X- or Y-sorted spermatozoa (Table 1). There was also no ram effect on the pregnancy rate within the flock.

Lambing rate

The number of ewes lambing, number of lambs born and sex of the lambs are presented in Table 2.

All lambs were born between Days 148 and 153 of gestation. There was no pregnancy loss between ultrasound pregnancy diagnosis at Day 60 and lambing. The number of lambs born per ewe lambing was similar after insemination with unsorted and sorted frozen–thawed spermatozoa.

Sex ratio

Twenty-five of the 26 lambs born from ewes inseminated with sex-sorted spermatozoa were of the predicted sex. There was a statistically significant difference in the sex of the lambs born from ewes inseminated with sorted X- (94.4% female) and Y- (100% male) chromosome-bearing spermatozoa compared with unsorted spermatozoa (51.3% male and 48.7% female; $P<0.05$). The proportion of male lambs born to ewes inseminated with Y-sorted spermatozoa and the proportion of female lambs born to ewes inseminated with X-sorted spermatozoa was indistinguishable from the re-sort analysis values obtained after each sorting session (Table 2).

Lamb weights

The mean birthweights for the lambs in both the sorted and control groups are presented in Table 3. Lambs in the control group had similar birthweights to lambs from ewes inseminated with X- or Y-sorted spermatozoa. Only the number of lambs born per ewe had a significant effect on the birthweights. Triplet or twin-born lambs had significantly lower birthweights than single lambs ($P<0.05$).

Discussion

This is the first report of the birth of lambs after insemination with low numbers of sex-sorted frozen–thawed

Table 2. Lambing after insemination of ewes with control and sex-sorted frozen–thawed spermatozoa

Type of semen	Number of ewes		Number of lambs		Sex ratio of lambs (%)	Sex ratio of sperm (%)
	Inseminated	Lambed (%)	Born (%)	Born/ewe lambing (± SEM)		
Control	48	26[a] (54.2)	39[a] (81.3)	1.5 ± 0.11	20:19[a] (51.3M:48.7F)	50.0[a] (expected-not tested)
X	48	12[b] (25.0)	18[b] (37.5)	1.5 ± 0.19	1:17[b] (94.4 F)	90.8 ± 0.15[b] X
Y	48	7[b] (14.6)	8[b] (16.7)	1.1 ± 0.14	8:0[b] (100.0 M)	94.2 ± 0.31[b] Y

Within columns, values with different superscripts differ significantly ($P<0.05$).

spermatozoa. The present study demonstrates that ram spermatozoa are capable of fertilization after being sex sorted and cryopreserved. It also shows that offspring of the predicted sex can be produced after insemination of only 1–2 × 10^6 motile (2–4 × 10^6 total) sex-sorted spermatozoa into the uterus by a standard commercial artificial insemination technique. The overall pregnancy rates obtained in this study were inexplicably low (54% control, 25% X-sorted group; 15% Y-sorted group) compared with pregnancy rates achieved by commercial artificial insemination programs (72%; Hill *et al.* 1998), though this may have been related to the relatively low fertility of one of the rams used. The pregnancy rate obtained for ewes inseminated with sorted frozen–thawed spermatozoa was less than half that of the controls. This may be a result of the low numbers of motile spermatozoa inseminated and/or the timing of insemination in relation to ovulation, as discussed below.

Fertilization rates are usually 20–30% lower after insemination with frozen–thawed than with fresh spermatozoa (Maxwell *et al.* 1993). The results of the present study suggest that spermatozoa that have undergone both sorting and cryopreservation may have lower fertilization rates than those that have only been frozen–thawed. However, it was not possible to distinguish between fertilization failure and embryonic loss in the present study, because egg fertilization rates were not determined. The low pregnancy rates obtained in this study may have been a result of the low numbers of spermatozoa inseminated, since the post-thaw motility of spermatozoa was similar for all treatment groups and the post-thaw acrosome status was higher for sorted than for unsorted spermatozoa.

There have been limited reports on the minimum numbers of fresh or frozen–thawed sorted spermatozoa required for effective fertilization after intrauterine or oviducal insemination in sheep. In our study, the overall pregnancy rate for both the X- and Y-sorted groups was 20% after insemination of 2–4 × 10^6 sorted frozen–thawed spermatozoa into either the uterus (26%) or UTJ (12%). Cran *et al.* (1997) reported the birth of six female lambs (including one set of twins) after the insemination of 25 ewes with 1 × 10^5 fresh X-sorted spermatozoa into the tip of the uterine horn. The minimum intrauterine insemination dose recommended by the sheep industry for frozen–thawed spermatozoa is 20–25 × 10^6 motile spermatozoa (Evans and Maxwell 1987). Results from several studies invcstigating the minimum effective intrauterine and/or oviducal insemination dose for fresh or frozen unsorted spermatozoa have been variable (reviewed by Evans 1988). These reports suggest that the insemination dose used in the present study was too low and that a dose in excess of 2–4 × 10^6 sex-sorted frozen–thawed spermatozoa might be required to achieve pregnancy rates closer to commercially acceptable levels.

Insemination of spermatozoa closer to the site of fertilization generally yields higher fertilization and pregnancy rates than inseminations carried out in the lower reproductive tract. This is particularly true when the inseminate dose is low or frozen–thawed spermatozoa are used (Jabbour and Evans 1991; Maxwell *et al.* 1993). However, in the present study, there was no significant difference found between insemination into either the uterus or UTJ with either sorted or unsorted frozen–thawed spermatozoa. Maxwell (1986*b*) suggested that the manipulation of the reproductive tract, which was necessary to inseminate into either the tip or base of the uterine horns, contributed to lower lambing rates than insemination into the middle of the uterine horn, which required minimal manipulation. Thus, pregnancy rates in the present study may have been compromised in ewes inseminated into the UTJ owing to the additional handling and manipulation of the upper reproductive tract compared with the simpler intrauterine insemination procedure.

In the present study ewes were inseminated 54–57 h after progestagen sponge removal. Previous studies have demonstrated higher egg fertilization and lambing rates when insemination of frozen–thawed spermatozoa occurred before, rather than after, ovulation (Maxwell 1986*a*). The median time of ovulation in ewes after synchronization of oestrus (with a progestagen sponge and PMSG) has been reported to be between 58 and 61 h (Maxwell 1986*a*). It may be that inseminations between 54 and 57 h were performed too early, relative to ovulation, and that the pregnancy rates for both UTJ and intrauterine inseminations could have been increased by inseminating just after ovulation at 60–64 h post sponge removal. Maxwell (1986*a*) and Eppleston *et al.* (1986) reported higher fertilization and lambing rates in non-superovulated ewes inseminated with frozen–thawed spermatozoa at 60 h compared with 48 h after sponge removal. This timing avoids the manipulation of the tract around the time of ovulation, which might interfere with ovum pick-up. In addition, the changes that occur in the oviduct after ovulation allow spermatozoa to pass imme-

Table 3. Mean (± SEM) birthweights of lambs and number of lambs born after insemination of ewes with control and sex-sorted frozen–thawed spermatozoa

Type of semen	Single		Twin		Triplet	
	Female (no. lambs)	Male (no. lambs)	Female (no. lambs)	Male (no. lambs)	Female (no. lambs)	Male (no. lambs)
Control	5.0 ± 0.19 kg (9)	4.5 ± 0.25 kg (5)	3.7 ± 0.18 kg (10)	3.9 ± 0.15 kg (12)	–	3.4 ± 0.53 kg (3)
X	5.4 ± 0.36 kg (6)	4.6 ± 0.00 kg (1)	3.1 ± 0.19 kg (8)	–	2.5 ± 0.41 kg (3)	–
Y	–	5.1 ± 0.26 kg (6)	–	4.0 ± 0.0 kg (2)	–	–

diately through the UTJ into the isthmus (Hunter *et al.* 1982). Given adequate capacitation conditions, these spermatozoa would participate in fertilization earlier than those deposited before ovulation, thus reducing the potential for embryonic loss associated with aged spermatozoa. This is particularly important when using frozen–thawed spermatozoa, which have approximately half the lifespan of fresh spermatozoa *in vivo* (reviewed by Salamon and Maxwell 1995; Gillan and Maxwell 1999). The capacitation status of the sorted frozen–thawed spermatozoa could not be determined in this study owing to quenching of chlortetracycline by the Hoechst stain used for determination of DNA content. A high proportion of the sorted spermatozoa were acrosome intact, as determined by FITC-PNA staining, but may have been capacitated, and thus have a short lifespan in the female reproductive tract. Therefore, timing of insemination relative to ovulation may also be an important influence on pregnancy rate.

For insemination to occur just after ovulation, the average time of ovulation in the flock needs to be determined. Walker *et al.* (1989*a*) and Eppleston *et al.* (1991) reported considerable variation in the time of ovulation in ewes synchronized with progestagen sponges and PMSG, with ovulation reported to vary over more than a 24-h period. However, when gonadotrophin-releasing hormone (GnRH) was incorporated into the synchronization regime, the period over which ovulation occurred was reduced to less than 12 h, depending on the time of GnRH injection after sponge removal. Despite the increase in synchrony of ovulation following the use of GnRH, there have been few reports of increased fertility after insemination with frozen–thawed semen (Maxwell 1986*a*; Walker *et al.* 1989*b*; Eppleston *et al.* 1991). However, an increased precision of the time of ovulation, and of insemination relative to ovulation, may play a more crucial role in obtaining satisfactory fertility after the use of sorted frozen–thawed spermatozoa, because the fertile lifespan of these cells in the female reproductive tract may be very short.

Embryonic loss in sheep is high even after natural insemination (Edey 1976). In the present study, the overall pregnancy loss between Days 18 and 60 (38.6) was not greater than that reported in ewes inseminated with fresh or frozen–thawed spermatozoa by other workers (e.g. Salamon and Maxwell 1995). In contrast to the pregnancy rate findings, there was no difference in pregnancy loss between the unsorted and sorted treatment groups. However, without fertilization data, we cannot say that normal embryonic development occurred after fertilization of oocytes with sorted frozen–thawed spermatozoa. *In vitro* studies in cattle (Lu *et al.* 1999) have shown similar fertilization rates but lower blastocyst rates (70%) with *in vitro* matured oocytes inseminated with sorted frozen–thawed spermatozoa compared with unsorted frozen–thawed spermatozoa. In trials involving the insemination of 1000 heifers with sorted frozen–thawed spermatozoa and unsorted frozen–thawed spermatozoa (Seidel *et al.* 1999), the overall pregnancy rate, determined by ultrasound, for heifers inseminated with sex-sorted frozen–thawed spermatozoa was 70–90% of those inseminated with unsexed frozen–thawed spermatozoa. Similarly, in the present study, there was no significant difference in pregnancy loss between the two insemination groups. However, because no fertilization or early embryonic data were collected, further *in vivo* studies would need to be carried out to determine fertilization rates and early embryonic loss potentially associated with sex-sorted frozen–thawed spermatozoa. It is more likely that the insemination of inadequate numbers of frozen–thawed sorted spermatozoa, either too early or too late relative to ovulation, contributed to the low fertilization rate.

The number of lambs born per ewe lambing was similar for ewes inseminated with Y-or X-sorted spermatozoa and control unsorted frozen–thawed spermatozoa. All of the lambs born from ewes inseminated with Y-sorted frozen–thawed spermatozoa were male and 94.4% of the lambs born to ewes inseminated with X-sorted frozen–thawed spermatozoa were female. This skewing of the sex ratio from the control, and expected, 50:50 ratio has been demonstrated previously in several species (reviewed by Garner 2001). The re-sort purity analysis values for spermatozoa obtained at the end of each sorting session were not significantly different from the observed sex proportions of the offspring produced by ewes inseminated with sorted spermatozoa. This supports previous findings by Welch and Johnson (1999) that re-sort analysis is a valuable tool for predicting the sex of offspring or the sex ratio of a given population after insemination with sex-sorted semen.

In conclusion, this study has demonstrated it is possible to obtain pregnancies with low numbers of sorted frozen–thawed ram spermatozoa using commercial artificial insemination techniques. However, the overall pregnancy rate for ewes inseminated with low numbers of sorted frozen–thawed spermatozoa was less than for ewes inseminated with commercial numbers of unsorted frozen–thawed spermatozoa. Further investigation is needed to determine the minimum effective dose of sorted frozen–thawed spermatozoa required to obtain commercially acceptable pregnancy rates. In addition, the optimum time to inseminate ewes relative to the time of ovulation remains to be determined. Overall fertility results are most likely to be improved by increasing the insemination dose. However, constraints on the number of spermatozoa that can be practically sorted should encourage further research on the control of the time of ovulation.

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SUCCESSFUL LOW-DOSE INSEMINATION BY A FIBEROPTIC ENDOSCOPE TECHNIQUE IN THE SOW

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A large volume insemination dose containing a large number of spermatozoa (2,000-3,000 million) is recommended to achieve good fertility results in sows. However, it is known that only 10 million spermatozoa are sufficient to obtain acceptable pregnancy rates when semen is introduced surgically into the uterotubal junction. Recently, we have developed a fiberoptic endoscope technique for deep uterine horn insemination, as close as possible to the uterotubal junction, without sedation of the female. With this technique, it is possible to pass the cervical canal and reach the depth of one uterine horn in 3-7 min in about 90% of the sows.

The objective of this study was to determine the effectiveness of inseminating sows with low numbers of spermatozoa using a fiberoptic endoscope technique under field conditions. Three sexually-mature hybrid boars of proven fertility and with satisfactory semen characteristics in the 10 wk preceding the experiment were selected as semen donors. Immediately after collection, sperm-rich fractions of ejaculates were diluted to 1,000 million spermatozoa/mL in BTS diluent. The sperm suspensions from the three boars were mixed and resuspended in BTS diluent to give insemination doses of 1,000, 200 or 50 million mixed sperm cells in 5 mL of diluent. Forty-six multiparous, commercial crossbred sows (parity 3-6) were used in the experiment. Twenty-four hours after weaning (approximately 3 to 4 wk after farrowing), all sows were injected with 1,250 IU eCG (Folligonan: Intervet International B.V., Boxmeer, The Netherlands) for stimulation of follicular growth. Ovulations were induced by im application of 750 IU hCG (Chorulon: Intervet International B.V., Boxmeer, The Netherlands) 72 h after the eCG injection. The animals were inseminated 36 h after hCG injection. Inseminations were performed using a modified artificial insemination spirette to produce a cervical lock and to assist in manipulating a specially designed flexible fiberscope (working length 1.35 m, outer diameter 3.3 mm, instrument channel 1.2 mm) which was inserted through the spirette and moved through the cervical canal into the uterine horn. Silicon spray was used to lubricate the spiral end of the spirette and the endoscope. If after entering one uterine horn we found difficulty in visualizing the entry of the other horn, unilateral insemination was performed. Semen samples (5 mL) were infused into one uterine horn by a syringe attached to the instrument channel of the fiberoptic endoscope. Then, 5 mL of BTS diluent was used to flush the remaining sperm suspension out of the fiberoptic endoscope. Sows were scanned with a 5 MHz sector scanner 24–28 d after insemination to determine the pregnancy rates and were managed until parturition to determine the litter size. Data were evaluated by chi-square test and ANOVA.

Pregnancy rates were 13/15 (86.6%), 16/18 (88.9%), and 12/13 (92.3%) for 1,000, 200 or 50 million sperm per insemination, respectively. Average litter sizes (mean ± SEM) were 9.61 ± 0.29, 9.75 ± 0.31 and 9.41 ± 0.38, respectively. Differences in pregnant rates and litter sizes were not significant. Further experiments are underway to determine critical sperm numbers to maintain a reasonable fertility level for biotechnologies such as sperm sorting where the number of viable spermatozoa is reduced.

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ABSTRACTS

Volume 2

A.I. IN SWINE: NEW STRATEGY FOR DEEP INSEMINATION WITH A LOW NUMBER OF SPERMATOZOA USING A NON-SURGICAL METHODOLOGY

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The new technologies applied to boar semen like sperm cryopreservation or sperm sorting for X/Y are associated with large changes in sperm when compared with fresh or diluted sperm. These changes, similar to those that occur during the sperm capacitation, produce weak sperms which are not able to produce high fertility rates, at least when traditional systems of A.I. are used. Besides, the flow sorting methodology produce a low number of sexed spermatozoa. In our opinion, there are two possibilities to improve this problem in order to apply these technologies to farm animals: 1) To increase the number and stability of sperm and 2) To develop new technologies that allow to apply these spermatozoa near to the uterotubal junction (UTJ) using a non-surgical procedure. Although it has been possible to inseminate near the UTJ in cattle and horses with a low number of spermatozoa, in other domestic species only surgical procedures have been possible. We have recently developed a technique for deep uterine horne insemination, as close as possible to the uterotubal junction, without sedation of the female. Briefly, the system is compound for a modified artificial insemination spirette to produce a cervical lock and to assist in manipulating a specially designed flexible fiberscope (working length 1.95 cm, outer diameter 3.3 mm and instrument channel 1.2 mm) which is inserted through the spirette and moved through the cervical canal into the uterine horn. With this technique, it is possible to reach the depth of one uterine horn in 3-7 min in about 90% of the sows. The sows (parity 2-6) were synchronized. 24 h after weaning, with 1.250 IU eCG and 750 IU hCG administered 72 h after eCG injection. In the first trial, we have used different concentrations (1000, 200 and 50 million of sperm in 5 ml of BTS) of fresh and diluted boar spermatozoa achieving pregnancy rates and litter sizes about: 65% and 9.5 respectively. Experiments are being developed to determine critical sperm numbers to maintain a reasonable fertility level using fresh and diluted boar spermatozoa.In the second trial, we have used cryopreserved sperm, decreasing the number of spermatozoa from 6 billions of sperm (usual dose) to 1 billion of sperm for insemination with the fiberoptic endoscope. We have obtained satisfactory results both in fertility rates and litter sizes. We are now determining the minimum number of cryopreserved sperm that we could be used to obtain these results, but preliminary result indicate that less than 1 billion. The use of the fiberoptic endoscope could be a useful tool to achieve high pregnancy rates decreasing the number of spermatozoa that are used with conventional procedures. Gender preselection or cryopreservation in swine could use this technology for practical application to farm animals.

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the dominant follicle at that time. Four of 10 cows in the ... treatment were observed to form a secondary CL compared to 1 of 10 controls. Overall, pregnancy rate was reduced ($P < .05$) from 53.5% in controls (n = 74) to 48.7% in the GnRH treatment (n = ... The GnRH treatment reduced pregnancy rate in virgin heifers ... and primiparous cows, whereas there was no effect in multiparous cows. A positive relationship existed between P4 and fertility, which was illared by GnRH treatment. For controls with P4 ≥4.3 ng/mL (median P4 concentration in all cows at the time of the second injection of $PGF_{2\alpha}$), pregnancy rate was higher ($P < .05$; 34.3 vs 40.5%) than that in GnRH-treated females of ... concentrations of P4 ≥4.3 ng/mL. In contrast, when P4 <4.3 ng/mL at the second injection, there was no treatment effect (54.3 vs 58.1%). Percentage of females with luteal phase concentrations of P4 at the time of the second injection was similar (95.2% vs 92.9%) in the GnRH treatment and control. The difference in pregnancy rate between treatments cannot be explained by ... of cycle or concentration of E2 at the ... ction of $PGF_{2\alpha}$. We concluded that ... the intervening week between injections ... heifers and first lactation cows, ... olden cows.

Key Words: Progesterone, $PGF_{2\alpha}$

Conceptus ... at ... > .10) of treatment, but days to conception ... than CON1 ewes (3.6 ± 1.2 vs 10.2 ... Ovulation rate, determined by laparoscopy ... born were not different ($P > .10$) between ... (1.4 ± .2 vs 1.5 ± .2 ovulations, and 1.2 ± ... respectively). The CIDR1 ewes lambed ... lambing season than CON1 ewes (9.5 ± ... lambing, respectively). In Trial 2, 14 ... CIDRs as in Trial 1 (CIDR2) and 14 ... injections of 15 mg PGF 10 d apart ... introduced on the day of CIDR remo... injection. The CIDR2 ewes exhibited asc... PG2 ewes (1.4 ± .4 vs 2.9 ± .4 d, respecti... to the synchronized estrus was 57.1% ... ewes. Progesterone concentration on d ... estrus was not different ($P > .10$) betw... (10.3 ± .3 vs 10.4 ± 3 ng/ml, respective... that estrus synchronization procedures ... tropics without adversely ... Timing ...

Key Words: Sheep, Estrus Synchron...

Sept 1 '95

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488 Insemination of Holstein heifers with very low numbers of unfrozen spermatozoa. G. E. Seidel, Jr.*, C. H. Allen†, Z. Brink, J. K. Graham, and M. B. Cattell‡. *Colorado State University, Fort Collins, †Atlantic Breeders Cooperative, Lancaster, PA, ‡DUO Dairy, Loveland, CO.*

The objective was to determine pregnancy rates when heifers were inseminated with very low numbers of sperm under ideal experimental conditions. Semen from three Holstein bulls was extended in Cornell Universal Extender plus 5% homologous seminal plasma to 1 x 10^6 or 2.5 x 10^6 sperm per 0.1 ml; 2.5 x 10^6 total sperm per 0.25 ml was used as a control. Fully extended semen was packaged in modified 0.25 ml plastic French straws to deliver the 0.1 or 0.25 ml inseminate doses. Semen was cooled to 5°C and used 26-37 h after collection. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse®) at 12-day intervals and inseminated with an embryo transfer straw gun and side-opening sheath into one uterine horn 24 h after detection of estrus. Insemination was ipsilateral to the side with the largest follicle determined by ultrasound 12 h after estrus; side of ovulation was verified by detection of a corpus luteum by ultrasound 7-9 days post-estrus. Pregnancy was determined by detection of a fetus by ultrasound 42-45 days post-estrus. The experiment was done in four replicates and balanced over three insemination technicians. Side of ovulation was determined correctly in 205 of 225 heifers (91%); surprisingly, pregnancy rates were nearly identical for ipsilateral and contralateral inseminates. Pregnancy rates were 38/93 (41%), 45/87 (52%), and 25/45 (56%) for 1 x 10^6, 2.5 x 10^6 and 2.5 x 10^6 sperm/inseminate ($P > .1$). There was a significant difference in pregnancy rates ($P < .05$) among technicians, but not among bulls. With the methods described, it may be possible to reduce sperm numbers per inseminate sufficiently that sperm sorted by sex with a flow cytometer would have commercial application.

Key Words: Semen, Insemination, Pregnancy

...90 ... Ovariectomy during the early ... not affect the ability of es... the negative feedback inhibition ... L. H. Anderson† and M. L. Day. The Columbus, OH.

Estradiol administration hastens ons... in estradiol negative feedback m... species. One action of estradiol ... number of neuronal synapses and neu... medial basal hypothalamus in prepu... hypothalamic region has been ... regulator of basal LH secretion, es... by augmenting neuronal development ... in estradiol negative feedback m... experiment is to determine if exposu... estradiol during the prepubertal p... occurrence of the prepubertal decl... feedback. Immature (n=5) and m... ovariectomized (OVX) at 227 ± 1... respectively. Blood samples were ... for 6 h from both the immature ... (MOVX) heifers, 340 days after O... serial sampling, heifers were ... implant (d 0). To ensure that a... the same concentration of estr... implant was adjusted according to ... cm/kg body weight). Serial blood ... on d 3 and 7 of treatment. No... observed in either LH pulse freq... h) or mean LH concentrations (ng... heifers on d -.5, 3 or 7. LH co... was higher ($P < .05$) on d 3 (1.34 ... (1.32 ± .08 vs .75 ± .15) in IMO... Thus, prepubertal exposure to ... appear to be a prerequisite for ... from the negative influence ... regulating LH secretion.

Key Words: Puberty, Estradiol, Ch...

513 Insemination of heifers with very low numbers of frozen spermatozoa. G. E. Seidel, Jr.[1]*, C. H. Allen[2], Z. Brink[1], M. D. Holland[1], and M. B. Cattell[2], [1]*Colorado State University, Fort Collins,* [2]*Atlantic Breeders Cooperative, Lancaster, PA,* [2]*DUO Dairy, Loveland, CO.*

The objective was to determine pregnancy rates when heifers are inseminated with extremely low numbers of frozen sperm under ideal field conditions. Semen from three Holstein bulls of above average fertility was extended in homogenized milk, 7% glycerol (CSS) extender plus 5% homologous seminal plasma to 2 x 10^5, 5 x 10^5 or 10 x 10^6 (control) total sperm per 0.25 ml French straw and frozen in moving liquid nitrogen vapor. Semen was thawed in 37°C water for 20 sec. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse®) twice at a 12-day interval and inseminated with an embryo transfer straw gun and side-opening sheath, half of the semen deep into each uterine horn 12 or 24 h after detection of estrus. The experiment was done in five replicates over 5 months, and balanced over two insemination technicians. Ambient temperature at breeding was frequently -10 to -20°C, so care was taken to keep insemination equipment warm. Pregnancy was determined by detection of a viable fetus using ultrasound 40-44 days post-estrus and confirmed 55-62 days post-estrus; 4 of 202 conceptuses were lost between these times. Day 55-62 pregnancy rates were 55/103 (53%), 71/101, (70%), and 72/102 (71%) for 2 x 10^5, 5 x 10^5 and 10 x 10^6 total sperm/inseminate (P<.02); there were no significant interactions (P>.1). Pregnancy rates were different (P<.05) among bulls (59, 62, and 74%), but not between technicians (64 and 65%) or insemination times post-estrus (65% for 12 h and 64% for 24 h, N=153 at each time). With the methods described, pregnancy rates in heifers were similar with 5 x 10^5 and 10 x 10^6 total sperm per inseminate.

Key Words: Frozen Semen, Insemination, Pregnancy

tozoa limits the potential to exploit stallions which produce semen that freezes badly, epididymal spermatozoa from castrated colts or sex-selected spermatozoa.

Materials and methods

Mares in this study were inseminated using the hysteroscopic insemination technique described by Morris et al. (2000). Fresh and frozen spermatozoa was used from twin Pony stallions. Epididymal spermatozoa was obtained from the testes of castrated colts and subsequently frozen.

Results and discussion

High conception rates of 60% (n = 10), 75% (n = 8) and 64% (n = 25) were achieved in mares inseminated videoendoscopically directly onto the papilla of the uterotubal junction ipsilateral to the ovary containing a ≥35 mm dominant follicle, respectively with 10, 5 or 1 million ejaculated spermatozoa extended in 100 μl sperm TALP. Conception rates fell to 29% (n = 14), 27% (n = 11) and 10% (n = 10), respectively, when the UTJ insemination dose was reduced to 0.5, 0.1 or 0.001 million spermatozoa. Nevertheless, conception rates of 44% (n = 9) and 17% (n = 6) were achieved in oestrous mares inseminated 30–32 h after hCG administration with 0.5 million motile spermatozoa that had been separated previously, by passage through a fluorescence activated cell sorter into their X- and Y-chromosome bearing populations. In the control group, 31% (n = 13) mares became pregnant after UTJ insemination with similarly low numbers of non-sorted spermatozoa that had been stored at room temperature for 5–8 h.

Satisfactory conception rates (47%, n = 34) were achieved when only 5 million motile frozen-thawed spermatozoa in 100 μl diluent were deposited directly onto the UTJ at a single fixed time of 30–32 h after hCG administration. However, when the spermatozoa were deposited just cranial to the cervix to simulate conventional artificial insemination, only 25% (n = 8) mares conceived. Low conception rates were achieved in mares inseminated by the UTJ method with 300 million (16%, n = 25) or 5 million (0%, n = 19) frozen-thawed epididymal spermatozoa.

In conclusion, hysteroscopic insemination enables extremely low numbers of spermatozoa to be deposited close to the site of fertilization, avoiding sperm losses that occur during conventional insemination.

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15

Pregnancy rates in mares following hysteroscopic or rectally-guided utero-tubal insemination with low sperm numbers

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Introduction

Pregnancy rates of mares were compared following insemination with 5 million total sperm deposited on or near the utero-tubal papilla by a rectally-guided approach, or by hysteroscopic

deposition of semen directly onto the utero-tubal papilla. In addition, the effect of intrauterine pretreatment with prostaglandin E_2 (PGE) deposited in the proximal uterine horn was evaluated.

Methods

Estrus was synchronized in 41 mares. Human chorionic gonadotropin (2500 IU, i.v.; Chorulon®, Intervet, Millsboro, DE, USA) was administered when mares had a dominant follicle ≥33 mm in diameter and they were inseminated the following day. Mares were assigned to 1 of 4 treatment groups. Mares in group PGE-HYS were infused with 0.25 mg PGE (in 1 ml saline) in the proximal uterine horn ipsilateral to the dominant follicle using a 65-cm insemination pipette 2 h prior to hysteroscopic insemination (HYS). HYS was done using a 1-m videoendoscope. The uterus was partially inflated with air and the videoendoscope was passed to the tip of the uterine horn, ipsilateral to the ovary with the dominant follicle. A pre-loaded catheter (Cook Veterinary Products, Bloomington, IN, USA) was advanced through the biopsy channel of the videoendoscope and semen was deposited on and around the utero-tubal papilla. Other treatment groups were: SAL–HYS (1 ml sterile saline was infused in the proximal uterine horn 2 h prior to HYS insemination); PGE–REC (PGE pretreatment 2 h prior insemination on or near the utero-tubal papilla, with semen placement verified by transrectal palpation of the insemination pipette); SAL–REC (saline pretreatment followed by rectally-guided insemination). Semen (25×10^6 ml^{-1}; 10% seminal plasma) had been previously stored in an Equitainer™ (Hamilton Thorn, Beverly, MA, USA) for 24 h following centrifugation and dilution in a milk–glucose extender. Insemination doses contained 5 million total sperm in a 200-μl volume. Mares were sedated immediately prior to insemination using detomidine (Dormosedan®, Pfizer, Lees Summit, MO, USA; 6 mg, i.v.) and butorphanol (Torbugesic®, Ft. Dodge Co., Ft. Dodge, IA, USA; 4 mg, i.v.), and were evaluated daily for ovulation. Pregnancy evaluations were done 12–14 days post-ovulation.

Results

Pregnancy rates (Table 1) were not different between mares inseminated using HYS (62%) or REC (50%) techniques. Pretreatment with intra-uterine PGE did not increase pregnancy rates (55%) compared to saline (57%).

Table 1
Pregnancy rates for mares inseminated with 5 million total sperm via HYS or REC following pretreatment with SAL or PGE

Treatment group	Pregnancy (%)
PGE–HYS	6/10 (60)
SAL–HYS	7/11 (64)
PGE–REC	5/10 (50)
SAL–REC	5/10 (50)
HYS insemination	13/21 (62)
REC insemination	10/20 (50)
PGE pretreatment	11/20 (55)
SAL pretreatment	12/21 (57)

Conclusions

Very low sperm numbers from a fertile stallion yielded acceptable pregnancy rates when mares were bred via REC or HYS. REC may be an acceptable alternative to HYS, especially when a suitable hysteroscope is unavailable.

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Interactions between seminal plasma and semen extender: effects on motility and fertility of centrifuged equine spermatozoa

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Introduction

This study evaluated motility and fertility of centrifuged equine spermatozoa which were cool-stored following dilution in a milk–glucose extender with or without supplemental Tyrode's medium (Padilla and Foote, 1991), as well as the effect of seminal plasma addition to extended semen.

Methods

Three ejaculates from each of three stallions were cooled for 48 h following centrifugation and resuspension in one of four different extenders: milk–glucose (E–Z Mixin CST; CST) extender containing 0% seminal plasma (CST-0); CST extender containing 20% seminal plasma (CST-20); CST extender supplemented with modified Tyrode's medium (65:35 (v/v)) and containing 0% seminal plasma (KMT-0); or KMT extender containing 20% seminal plasma (KMT-20). Sperm motility (percentage motile sperm (MOT), percentage progressively motile sperm (PMOT), curvilinear velocity (VCL), and straight-line velocity (VCL)) were evaluated using a computerized system. Estrus was synchronized in 35 mares. When the dominant follicle reached a 33-mm diameter, hCG (Chorulon®, Intervet, Millsboro, DE, USA; 2500 IU, i.v.) was administered. After 24 h, mares were inseminated with 250 million total sperm (50×10^6 ml^{-1}) which were previously stored in an Equitainer™ (Hamilton Thorne, Beverly, MA, USA) for 48 h following centrifugation and processing in CST-20 or KMT-0. Pregnancy evaluations were done 14–16 days post-ovulation.

Results

Mean VCL and VSL were greater ($P < 0.05$) in CST-20 compared with CST-0 (Table 1). Conversely, mean values for all motility variables were greater in KMT-0 than MKT-20 ($P < 0.05$). Mean MOT and VCL were greater in KMT-0 compared to CST-20 ($P < 0.05$). Nonetheless, pregnancy rates were similar ($P > 0.05$) for mares bred using CST-20 (13/18; 72%) or KMT-0 (13/17; 76%).

Conclusions

KMT extender may be useful for cooled transport of semen from stallions in which all seminal plasma must be removed because of suspected toxic effects of seminal plasma on spermatozoal viability. Addition of Tyrode's medium may be detrimental to sperm motility when seminal plasma is present.

BOAR SEMEN PRESERVATION IV

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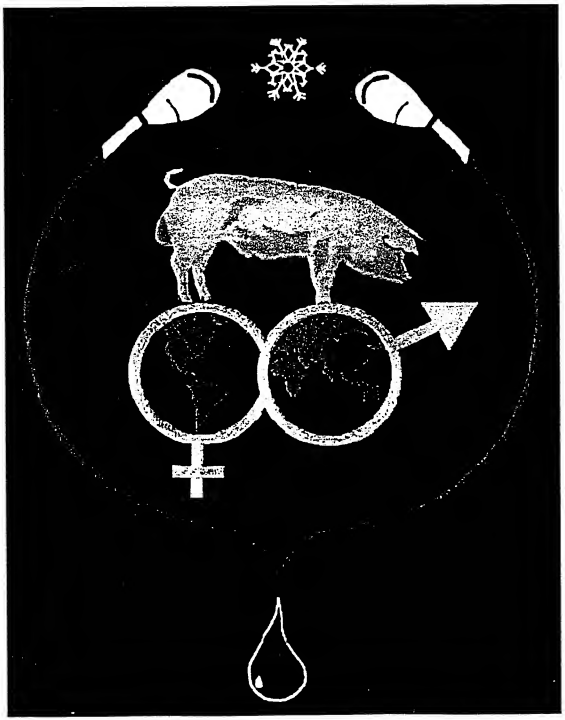

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Low Dose Insemination Technique in the Pig

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Abstract

The aim of the experiment was to determine the minimally necessary number of sperm present at the UTJ for normal fertilization and litter size. Estrus cycle was induced hormonally in prepuberal gilts. Exactly 32 or 38 h after hCG injection or at the time of ovulation gilts were inseminated surgically into the tip of the uterine horn with 1×10^9, 2×10^8, 2×10^7, 1×10^7 or 2×10^6 sperm/animal. The insemination volume was 0.5 ml. No differences were seen between the first four groups in fertility rate, farrowing rate and litter size. However, a 50% reduction in fertility was found at the lowest sperm concentration. Sows at first and second parity were checked for heat with a teaser boar. At 32 h after onset of estrus signs, sows were inseminated surgically with 1×10^9 or 2×10^8 sperm/ animal. The dosage volume was also 0.5 ml. No significant differences were seen for pregnancy rates however the litter sizes dropped. Regular single insemination with 3×10^9 and $1x10^9$ sperm didn't differ in pregnancy rate and litter size from the surgical insemination results. Prepuberal gilts were prepared as above. Gilts were slaughtered 48h after surgical insemination. Reflushing of embryos at day 2 after insemination showed no differences between high and low sperm concentration. Embryo morphology didn't differ. The developmental potentials of the embryos were tested under an inverted microscope in a time lapse analysis system. Data are just under investigation. So far, no differences were found in the developmental pattern of embryos. In conclusion, low sperm concentration and low dosage volume are sufficient for successful insemination, if sperm are deposited close to the UTJ. As shown in cattle and horse this technique is a useful way to inseminate sexed semen.

Introduction

In the recent years pig AI has become more and more interest and the number of first inseminations has increased rapidly, especially in the USA but also in Europe and Asia. As a consequence the demand for semen grew very quickly. This raises the question whether ejaculates may be used more efficiently in order to satisfy the semen market and still run intensive genetic selection programs to produce top genetic boars. Additionally, the advent of sexed semen requires an alternative means of transporting sperm to the site of fertilization since production rates of X- and Y-chromosome bearing sperm are low (Johnson, 1991). Even with the recent increase in sexed sperm production rates (Johnson, this volume; Johnson and Welch, 1999) the number of sperm required for fertilization in the pig by AI far exceed those that can be sorted in a few hours. Such (sexed) sperm populations are very valuable and use has to be made as efficiently as possible. One major reason for semen shortage is that other than in cattle AI, advantage of boar ejaculates have not been used to their maximal extent. Usually 2 to 4 billion sperm are inseminated twice or even three times in the estrous cycle of a gilt or sow, which means in total up to 12 billion sperm are used for one pregnancy. In cattle AI for example only 10 to 20 million sperm are inseminated (Colenbrander 1991). As compared to an insemination volume of 0.25 to 0.5 ml in cows the volume of an AI dose for pigs is relatively large containing 80 to 100 ml. A certain ratio of liquid volume and number of sperm cells seems to be necessary (Stratman et al. 1959; Paredis 1962).

What is the reason for such large volume and the high sperm number per insemination? Firstly, boars are "uterus inseminators". Their physiological ejaculate volume is much larger then in ruminants and sperm are not that concentrated. Secondly, the sow is an multiparous animal with long uterine horns and sperm have to make their long way from the site of insemination to the oviductal ampulla to meet several fertilizable oocytes. What counts economically in the pig is not a pregnancy, its the litter size and as many oocytes as possible have to be fertilized. Thirdly, growth of follicles and time of ovulation can not tested directly for example by rectal palpation. Ultrasonography may be a solution in the future, but so far only secondary signs of estrus are used as indicators to determine the onset of heat. From this time point on, any other event in the cycle is only estimated and varies between animals, breeds, farms etc. Therefore, it makes sense to inseminate more than once per cycle to give pregnancy a maximal chance (Reed 1982; Flowers & Esbenshade 1993).

Biological fate of sperm on their way to the oocyte

After insemination sperm pass through the uterine body, the uterine horns and the utero-tubal junction into the oviduct. In the ampulla of the oviduct sperm cells meet the fertilizable oocytes and penetrate the zona pellucida. Such sperm transport is a complex process, which is effected by several factors. It

begins with the stimulation of the female partner through mating or insemination itself, depends on the composition of the ejaculate or the inseminate, is effected by the activity and secretions of the female genital tract, and finally but most important is regulated by hormones and immunogenic factors (Bower 1974; Einarsson 1980; Viring 1981; Claus et al. 1979; Drobnis and Overstreet, 1993). Important is the direction of the myometrial waves that transport the inseminate towards the utero-tubal junction (UTJ). The direction of these waves depends on the status of the estrous cycle (Zerobin 1968). Intensive contractility of the uterus directly after mating is responsible for a rapid sperm transport to the oviduct (Bower, 1974) but this first wave contains not the sperm population that fertilizes. These sperm cells are not capacitated, their viability and membranes are weak and at least in the rabbit most of them are pushed directly through the oviduct into the abdominal cavity before they meet any fertilizable oocyte (Overstreet 1983; Overstreet & Cooper 1978).

In the second phase of sperm transport, sperm cells are moved much slower through the porcine female tract. On their way they are surrounded by substances of the seminal plasma which prevent the sperm cells to undergo capacitation too early (Hunter et al. 1998; Yanagimachi 1994). Once the sperm have reached the UTJ, these factors are not longer present and capacitation can start. For example AWN-1 was not detectable at the UTJ anymore 18h after insemination (Calvete et al 1997). At the UTJ and the distal part of the oviductal isthmus a sperm reservoir is build up. Here sperm cells are very selectively prepared for their final migration to fertilize the oocytes in the oviduct (Viring et al. 1980; Hunter 1981, 1984; Yanagimachi 1994). The reservoir is important to maintain the viability and integrity of sperm for about 24 to 42 h (Hunter 1988). Mburu (1997) showed that boar sperm are located at specific regions of the UTJ and the distal oviduct. Only sperm that had close contact to the ciliary epithelial cells had intact membranes and were classified as fertile. Similar observation were seen in vitro when intact ciliary epithelial cells were able to bind sperm (Suarez et al. 1991). It seems that only non-capacitated sperm bind to the epithelial cells (Fazeli et al. 1999). Only a few sperm are released and reach the oocytes, eventually guided by a temperature gradient (Hunter & Nichol 1986; Hunter 1995). After 2 days the number of sperm at the UTJ decreases gradually (Polge 1978; Viring & Einarsson 1981).

Sperm losses after mating or insemination

Transportation through the uterus and the selection at the UTJ diminishes the number of sperm extremely and only a small proportion enters the zona pellucida. Additionally, back flow of sperm and seminal plasma/extender after mating or insemination is a major reason for sperm losses. Although the bulbourethal secretions close the cervix a the end of ejaculation and remain there for about two hours (Lovell & Getty 1968) 25–30% of sperm and up to 70% of the volume are excreted retrogradely (Viring & Einarsson 1981; Steverink et al. 1998). In the bovine such back flow was also observed (Mitchell et al. 1985) and it was irrelevant for the amount of back flow whether the semen was deposited into the uterine corpus or horn or cranial cervix. In contrast insemination into the caudal part of the cervix increased the amount of back flow significantly (Gallagher and Senger 1989). At least in the pig the backflow seems to be a physiological event. Increased back flow rates were only effective if the total number of sperm was lower than 1×10^9 per 80 ml (Steverink et al. 1998). If the backflow occurred not at insemination but 0.5 to 2.5 hours later, no effects were seen for the establishment of a sufficient sperm reservoir at the UTJ. All trials to prevent the backflow with a tamponade in the cervix failed to increase the sperm number in the uterus (Pursel 1982).

Further sperm losses are caused by adhesion of sperm to ciliary epithelial cells of the uterus and migration into uterine glands. Additionally, the sperm population is attacked within 30 minutes after insemination when polymorphonuclear leukocytes occur in the uterine lumen (Lovell & Getty 1968) and can be found there for about 9 to 10 hours (Hadjisavas et al. 1994). The fluid of the inseminate is reduced much quicker. About two hour after insemination only foamy remnants can be found in the tip of the uterine horn.

Deep intrauterine insemination

The implication of the above mentioned mechanisms is to reduce the losses of sperm on their way to the oocytes. This can be done by preventing back flow and rapid sperm transportation, protection of the sperm cells against immunological reactions or placing the sperm cells closer to the utero-tubal junction. The objective of some basic experiments that we recently performed in gilts and sows was to identify the minimal number of sperm cells that are required to be inseminated into the tip of the uterine horn without losing the fertilizing potentials of the inseminate.

In cows significantly increased fertility rates were obtained when semen was introduced into the uterine horn instead of placing it into the uterine body (Zavos et al. 1985; Senger et al. 1988; Williams et al. 1987; Lopez-Gatius & Camon-urgel. 1988; Dalton et al. 1999). Seidel et al. (1997) showed that it is possible to reduce the sperm number per insemination and used 2×10^8 living sperm cells for deep intrauterine insemination of sorted sexed bull semen. The percentage of the pregnancy rates did not differ significantly from the controls. Buchanan et al. (1999) showed that pregnancies can be obtained in horses after deep intrauterine insemination, but pregnancy rates were diminished. In dogs intrauterine insemination with frozen-thawed semen gave better results than insemination into the vagina. (Fontbonne & Badinand 1993; Wilson 1993).

Polge et al. (1970) obtained high fertilization rates (90%) in pigs when about 10 million frozen thawed sperm in 0.5 ml were directly inseminated into the oviduct six hours prior to

estimated time of ovulation. Similar results were found by Soenbeck and Didion (1995) under farm conditions when they inseminated frozen thawed semen into the oviduct (pregnancy rate 29%; litter size 5.9 living piglets). Piglets have been also been produced after insemination of about 200,000 sexed sperm directly into the oviduct (Johnson et al. 1991).

The disadvantage of an insemination protocol directly into the oviduct is that even under long term aspects it will not be possible to reach this location non-surgically. In contrast first trials to introduce a catheter system into the uterine horn and push it forward to the tip are promising (Martinez et al. 2000) and it is a challenge to overcome the special anatomical conditions in female pig where the long cervix has tight cushions and can hardly be passed in young gilts.

Our own experiments were performed surgically (Krüger et al 1999; Krüger & Rath 2000). Prepuberal gilts were hormonally stimulated with PMSG and hCG and were inseminated with 10^9, 10^8, 10^7 5×10^6 and 10^6 sperm per uterus horn at 38 h and 32 h after hCG treatment or at the time of ovulation. Pregnancy rates, farrowing rates and litter size did not differ significantly between these groups as long as more than 10^6 sperm were used in 0.5 ml of extender. Partly, gilts were not allowed to go to term, but were slaughtered 48 h after insemination and embryos were re-flushed. These embryos were investigated for their integrity and were then cultured in vitro for five days to learn about their developmental potentials. The reason for this part of experiment was that after low dose insemination sperm with non-compensable deficiencies might have fertilized the oocytes (Saacke et al. 1998), because competition of sperm cells at least might be diminished at such low sperm concentrations. We found no indications for this with as long as the inseminated sperm number was higher than 10^6 sperm with the boars we used. But it has to be kept in mind that the results may differ if other boars are used. In this part of our experiments the lowest sperm dosage was again not fully competent and significantly less oocytes were fertilized as compared to the other groups. Additionally, embryos derived in the lowest concentration group had less developmental competence as their cell cycles were prolonged from the second cell cycle onwards and none of the embryos in this group developed to hatching blastocysts. This may indicate that sperm with non-compensable defects participated in fertilization.

In the experiment with the stimulated gilts, litter sizes of all groups were lower as could be expected from normal insemination (−2.2 piglets in average). It was not dependant on sperm dosage and not on the time of insemination. But there are several possibilities for this like the age of the gilts, the hormonal stimulation and the surgical intervention. In order to exclude hormonal and age effects the experiment was repeated with sows after weaning and 10^9, 10^8 and 10^7 sperm were inseminated per horn surgically. Additionally two groups were inseminated once intra-cervically (regular insemination) with 10^9 or 3×10^9 sperm. Data between all groups didn't differ significantly and litter sizes were similar to what could be expected after regular insemination.

Conclusion

Deep intra-uterine insemination in pigs is a possibility to reduce the number of sperm necessary for insemination. We have learned that 5×10^6 sperm inseminated in the tip of the uterine horn are sufficient for normal fertilization. These data are based on specific boars. In general, sire effects will have to be observed very carefully. At the moment no device is available to introduce this technique into the field. But major technical improvements are foreseeable. Additionally, research will focus on lower locations in the uterus to make non surgical insemination protocols easier to handle.

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Fertility of Superovulated Ewes after Intrauterine or Oviducal Insemination with Low Numbers of Fresh or Frozen–Thawed Spermatozoa

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Abstract

Two experiments investigated the effects of number of spermatozoa inseminated and time and site of insemination of fresh and frozen–thawed semen on fertility in Merino ewes treated with intravaginal progestagen pessaries and a combination of pregnant mare serum gonadotrophin and follicle stimulating hormone. In Experiment 1, ewes were inseminated in the uterus or oviducts with totals of 10^4, 10^5, 10^6 or 10^7 fresh spermatozoa 44 or 68 h after pessary removal. Ova recovered 48 h later were classified as fertilized if they had cleaved. Proportion of fertile ewes (ewes with fertilized ova per ewes inseminated) and fertilization rate of ova (ova fertilized per ova recovered) were higher for inseminations 44 h (63% and 45%) than 68 h (38% and 19%) after pessary removal ($P<0{\cdot}001$). More ewes were fertile after oviducal (61%) than after intrauterine insemination (39%; $P<0{\cdot}05$), and with high (10^6 and 10^7) than with low (10^4 and 10^5) doses of spermatozoa for intrauterine (54% *v.* 24%; $P<0{\cdot}05$) but not for oviducal inseminations (63% *v.* 59%). Fertilization of ova was better after oviducal than after intrauterine inseminations (44% *v.* 22%; $P<0{\cdot}001$), and with high than with low doses of spermatozoa (45% *v.* 21%; $P<0{\cdot}001$). In Experiment 2, ewes were inseminated with 10^5, 5×10^5 or 10^6 total fresh or frozen–thawed spermatozoa in the uterus or oviducts, 44 h after pessary removal. Proportion of fertile ewes and fertilization rate of ova were higher for oviducal (75% and 59%) than for intrauterine inseminations (27% and 14%; $P<0{\cdot}001$), and for fresh (56% and 35%) than for frozen–thawed semen (47% and 29%; $P<0{\cdot}05$). Fertilization rate of ova was higher for inseminate doses of 5×10^5 and 10^6 than for 10^5 total spermatozoa (36% *v.* 25%; $P<0{\cdot}01$). The study showed that fertilized ova can be obtained after deposition of 10^6 fresh spermatozoa in the oviducts of superovulated ewes, and that the minimum effective dose is between 10^5 and 10^6 fresh spermatozoa.

Introduction

The fertilization rate of ova following superovulation is of major importance in the production of viable embryos for embryo transfer programmes (see Maxwell *et al.* 1990). Fertilization is generally low following vaginal or cervical insemination of superovulated ewes (Trounson and Moore 1974; Evans *et al.* 1984), but this can be overcome by depositing semen closer to the site of fertilization (in the uterus by laparotomy, Trounson and Moore 1974; in the uterus by laparoscopy, Killeen and Caffery 1982; or in the oviducts by laparotomy, Killeen 1969; Jabbour and Evans 1991). Fertilization of ova is also influenced by the time of insemination (Evans *et al.* 1984; Jabbour and Evans 1991) and type of semen (fresh or frozen–thawed, Armstrong and Evans 1984; Hunton *et al.* 1986; Jabbour and Evans 1991).

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There have been limited studies on the effect of number of spermtozoa on the fertilization rate of ova. In non-superovulated ewes, satisfactory pregnancy rates have been reported following intrauterine insemination with minimum doses of $12{\cdot}5\times10^6$ total (Davis *et al.* 1984), or 25×10^6 motile spermatozoa (Maxwell 1986), whereas unsatisfactory fertility was reported with intrauterine inseminate doses of 1 or 6×10^6 (Walker *et al.* 1984) and $0{\cdot}5$ or 5×10^6 motile spermatozoa (Maxwell 1986).

Walker *et al.* (1984) reported no difference in the fertilization rates of ova when either 2 or 20×10^6 motile fresh or frozen-thawed spermatozoa were deposited by intrauterine insemination in superovulated ewes. However, Jabbour *et al.* (1988) recommended that doses in excess of $6{\cdot}2\times10^6$ motile sperm were probably necessary if frozen-thawed semen was to be used in superovulated ewes.

The aim of the present study was to examine the effects of time of intrauterine and oviducal insemination on the fertility of superovulated ewes, and to determine the minimum numbers of fresh and frozen-thawed spermatozoa that could be used for intrauterine and oviducal insemination. Preliminary reports on this work have been presented elsewhere (Maxwell *et al.* 1992*a*, 1992*b*).

Materials and Methods

Mature Merino ewes maintained under field conditions at Armidale, NSW, were used in two experiments carried out during autumn (March–May) 1992, when the ewes were exhibiting regular oestrous cycles. The time of oestrus and ovulation was controlled by progestagen-impregnated intravaginal pessaries (30 mg Chronogest, Intervet, Lane Cove, NSW) which were inserted for 12 days. At 48 h before pessary removal ewes were given a single intramuscular injection of 300 I.U. pregnant mare serum gonadotrophin (PMSG, Pregnecol; Horizon Animal Reproduction, North Ryde, NSW) and either 8 mg (Experiment 1) or 6 mg (Experiment 2) follicle stimulating hormone (FSH, Ovagen; Horizon Animal Reproduction, North Ryde, NSW) to induce superovulation. The ewes received 50 μg gonadotrophin releasing hormone (GnRH, Fertagyl; Intervet, Lane Cove, NSW) 24 h after pessary removal.

Experiment 1

At 44 or 68 h after pessary removal, 102 ewes were inseminated in the uterus or oviducts with inseminate doses of 10^4, 10^5, 10^6 or 10^7 total fresh spermatozoa, split between both sides of the reproductive tract.

Experiment 2

At 44 h after pessary removal, 112 ewes were inseminated in the uterus or oviducts with inseminate doses of 10^5, 5×10^5 or 10^6 total fresh or frozen-thawed spermatozoa, split between both sides of the reproductive tract.

Preparation of Semen and Insemination

Pooled semen from three Merino rams, collected with an artificial vagina, was used in both experiments. The semen was collected within 6 h of insemination and assessed for motility and concentration of spermatozoa. In Experiment 2 the pooled semen was split into two parts for use fresh (A) or after freezing (B). Part B was diluted 1:2 (semen:diluent, v/v) with a Tris–egg yolk–glycerol diluent and then frozen in pellet form as described by Evans and Maxwell (1987). The frozen pellets were stored in liquid nitrogen for 1 h, then thawed in dry tubes at 37°C. The fresh and frozen-thawed semen was diluted with Dulbecco's phosphate-buffered saline (PBS; Flow Laboratories, North Ryde, NSW; supplemented with 4 g L^{-1} BSA, 1 g L^{-1} glucose and 25 mg L^{-1} Kanamycin) to give the appropriate numbers of spermatozoa in an $0{\cdot}2$ mL inseminate dose. All semen was held at 37°C until insemination.

All inseminations were carried out under general anaesthesia induced by Xylazine (4–6 mg per ewe, i.m.; Rompun, Bayer, Botany, NSW) and Ketamine (100–200 mg per ewe, i.m.; Parnell Laboratories, Silverwater, NSW). Intrauterine inseminations were conducted by laparoscopy as described by Killeen and Caffery (1982), and $0{\cdot}1$ mL of diluted semen was injected into the lumen of each uterine horn. Oviducal inseminations were carried out by laparotomy and $0{\cdot}1$ mL of diluted semen was injected into each oviduct via the infundibulum through a disposable 10-μL capillary pipette (Unopette: Becton Dickinson, Rutherford, New Jersey).

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Recovery and Evaluation of Embryos

At 48 h after insemination, the ewes were killed by captive bolt and their reproductive tracts removed. Ova were recovered within 15 min of death by flushing the uterine horns and oviducts with PBS. The ova were located by light microscopy and those that had cleaved were classed as having been fertilized. The numbers of recent ovulations were recorded at the time of ova flushing.

Statistical Analysis

Data on the number of recent ovulations, number and proportion of ova recovered, and number and proportion of ova fertilized were subjected to least-squares analysis of variance (Harvey 1990). Between- and within-treatment comparisons of proportions of ewes with fertilized ova were made by χ^2 analysis.

Results

Experiment 1

None of the treatments affected the number of recent ovulations observed at the time of ova recovery (mean $14{\cdot}1 \pm 0{\cdot}88$), the number (mean $7{\cdot}8 \pm 0{\cdot}56$) or proportion of ova recovered (overall recovery rate 798/1432, 56%). There were significant effects of time of insemination, site of insemination and dose of inseminate on the proportion of fertile ewes (ewes with fertilized ova per ewes inseminated) and the ova fertilization rate (ova fertilized per ova recovered). The proportion of fertile ewes and the fertilization rate of ova were higher for inseminations 44 h (31/49, 63% and 183/411, 45%) than 68 h (20/53, 38% and 73/387, 19%) after pessary removal ($P<0{\cdot}001$).

As there were no interactions between time of insemination and any of the other treatments, the data for site of insemination and dose of inseminate pooled for time of insemination are presented in Table 1. More ewes were fertile after oviducal than after intrauterine insemination ($P<0{\cdot}05$), and for high (10^6 and 10^7) than for low (10^4 and 10^5) doses of spermatozoa for intrauterine ($P<0{\cdot}05$) but not oviducal inseminations. Fertilization of ova was better after oviducal than after intrauterine inseminations ($P<0{\cdot}001$), and for high than for low doses of spermatozoa ($P<0{\cdot}001$).

Table 1. Fertile ewes (No. of ewes with fertilized ova/inseminated) and fertilization of ova (No. of ova fertilized/recovered) after oviducal and intrauterine insemination of superovulated ewes with different doses of fresh spermatozoa

Site of insemination	Dose (total sperm)	Fertile ewes (%)	Fertilization of ova (%)
Oviducts	10^4	8/12 (67)	42/118 (36)
	10^5	7/12 (58)	26/82 (32)
	10^6	9/13 (69)	41/72 (57)
	10^7	7/14 (50)	53/93 (57)
Uterus	10^4	2/12 (17)	3/106 (3)
	10^5	4/13 (31)	18/123 (15)
	10^6	8/13 (62)	47/108 (44)
	10^7	6/13 (46)	26/96 (27)

Experiment 2

None of the treatments affected the number of recent ovulations observed at the time of ova recovery (mean $11{\cdot}2 \pm 0{\cdot}66$). However, the number ($P<0{\cdot}01$) and proportion of ova recovered ($P<0{\cdot}001$) were lower for oviducal (least-squares mean $5{\cdot}5 \pm 0{\cdot}67$ and 309/642, 48%) than for intrauterine inseminations (least-squares mean $8{\cdot}2 \pm 0{\cdot}68$ and 449/613, 73%).

The proportions of fertile ewes and fertilized ova for this experiment are presented in Table 2. Proportions of fertile ewes and fertilized ova were higher for oviducal (43/57, 75% and 184/313, 59%) than for intrauterine insemination (15/55, 27% and 62/449, 14%; $P<0{\cdot}001$), and for fresh (31/55, 56% and 131/372, 35%) than for frozen–thawed semen (27/57, 47% and 115/390, 29%; $P<0{\cdot}05$). Overall, dose of inseminate did not affect the proportion of fertile ewes, but fertilization rate of ova was higher for inseminate doses of 5×10^5 and 10^6 than for 10^5 total spermatozoa (180/501, 36% *v.* 66/261, 25%; $P<0{\cdot}01$).

Table 2. Fertile ewes (No. of ewes with fertilized ova/inseminated) and fertilization of ova (No. of ova fertilized/recovered) after oviducal and intrauterine insemination of superovulated ewes with different doses of fresh and frozen–thawed spermatozoa

Site of insemination	Dose (total sperm)	Fertile ewes (%) Fresh	Fertile ewes (%) Frozen	Fertilization of ova (%) Fresh	Fertilization of ova (%) Frozen
Oviducts	10^5	7/9 (78)	5/9 (56)	38/61 (62)	22/50 (44)
	5×10^5	6/9 (67)	7/10 (70)	21/28 (75)	28/59 (47)
	10^6	8/10 (80)	10/10 (100)	31/42 (74)	44/73 (60)
Uterus	10^5	3/8 (38)	0/9 (0)	6/94 (6)	0/56 (0)
	5×10^5	4/9 (44)	1/9 (11)	24/69 (35)	2/57 (4)
	10^6	3/10 (30)	4/10 (40)	11/78 (14)	19/95 (20)

Discussion

Both intrauterine and oviducal insemination have been reported to overcome the low fertility associated with superovulation of ewes with exogenous gonadotrophins (Trounson and Moore 1974; Armstrong and Evans 1984; Jabbour and Evans 1991). The present study confirmed the importance of site of insemination and time of semen deposition relative to progestagen withdrawal. It also showed that fertilized ova can be obtained after deposition of 10^4 spermatozoa in the oviducts of superovulated ewes. However, the overall fertilization rates obtained in this study were low, and may have been limited by the conditions of the experiment. In addition, the cleavage of ova 48 h after insemination may not be a reliable indicator of their subsequent viability.

The lowest previously published inseminate dose for superovulated ewes was 2×10^6 spermatozoa deposited in the uterus, which yielded ova fertilization rates of 39 and 60% for frozen–thawed and fresh semen respectively (Walker *et al.* 1984). The minimum effective inseminate dose for oviducal insemination in our study was between 10^5 and 10^6 total spermatozoa for fresh semen, and at least 10^6 total spermatozoa for semen that had been frozen–thawed (Table 2).

The ova fertilization rates obtained in our study after intrauterine insemination were low (22 and 14% in Experiments 1 and 2 respectively), although fertilization rates similar to those reported by Walker *et al.* (1984) were obtained in some experimental treatments (see Tables 1 and 2). It may be that intrauterine inseminate doses in excess of 10^6 spermatozoa are generally required to obtain adequate ova fertilization. Alternatively, the general anaesthetic procedure used in the present study may have interfered with sperm transport and/or viability in the reproductive tract, as intrauterine insemination by laparoscopy is normally conducted under local anaesthesia. No explanation can be given for the relatively low fertilization of ova obtained after intrauterine insemination of ewes with 10^7 spermatozoa in Experiment 1.

Ova fertilization rates are usually 20–30% lower with frozen than with fresh semen (Hunton *et al.* 1986), particularly when low doses of frozen–thawed spermatozoa ($<10\times10^6$ motile) are deposited (Jabbour *et al.* 1988). Jabbour *et al.* (1988) obtained higher rates of

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) zen	Fertilization of ova (%) Fresh	Frozen
(56)	38/61 (62)	22/50 (44)
(70)	21/28 (75)	28/59 (47)
(100)	31/42 (74)	44/73 (60)
(0)	6/94 (6)	0/56 (0)
(11)	24/69 (35)	2/57 (4)
(40)	11/78 (14)	19/95 (20)

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fertilization with $6{\cdot}2\times10^6$ and $3{\cdot}1\times10^6$ fresh motile spermatozoa (14/15, 93% and 23/23, 100%) than with the same doses of frozen-thawed semen (14/32, 44% and 21/31, 68%) deposited in the uterus, and recommended that doses in excess of $6{\cdot}2\times10^6$ motile sperm were probably necessary if frozen-thawed semen was to be used in superovulated ewes. We were unable to confirm an optimum inseminate dose range for frozen-thawed spermatozoa in the present study. However, we were able to obtain 60% of ova fertilized when 10^6 frozen-thawed spermatozoa (equivalent to 3×10^5–5×10^5 motile spermatozoa) were deposited by oviducal insemination, suggesting that inseminate doses lower than those recommended by Jabbour *et al.* (1988) may be effective if placed in the oviducts.

High fertilization rates, and therefore high yields of embryos, are also dependent on the time of insemination, and that timing often influences both fertilization and ova recovery rates. Recovery rates are generally lowest, but fertilization highest, when intrauterine inseminations are performed close to the time of ovulation (Walker *et al.* 1989; Hunton *et al.* 1986).

In the present study, two times of insemination were examined in an attempt to compare the effects of aged spermatozoa (insemination before ovulation) with aged oocytes (insemination after ovulation) in superovulated ewes. We timed our inseminations to be approximately just before or after ovulation. The 44-h insemination time was chosen as equal to (20–25 h after induced preovulatory luteinizing hormone (LH) surge: Cumming *et al.* 1971) or just before the estimated time of ovulation (42–54 h after progestagen withdrawal: Maxwell *et al.* 1990, Ryan *et al.* 1992). The majority of ovulations would have been completed by the time of the 68-h inseminations (Ryan *et al.* 1992). This was supported in Experiment 1 by an observation of the number of recent ovulations at the time of insemination and at ova recovery; approximately 30% of follicles had ovulated at 44 h compared with 90% at 68 h after progestagen withdrawal. However, it was not possible to distinguish between unovulated and supernumerary follicles at the time of insemination.

In our study, recovery rate was not affected by insemination time, but fertilization rates were lower for inseminations performed 68 h compared with 44 h after pessary removal. Robinson *et al.* (1989) obtained higher fertilization rates at 48 and 60 h than at 36 h, but Walker *et al.* (1984) reported no difference in ova fertilization rate when intrauterine insemination was undertaken at 54 or 64 h after progestagen withdrawal. However, these differences in fertility may be partly attributable to variation in the precision and timing of ovulation associated with the different superovulation regimes. The use of GnRH may improve the synchrony of ovulation (Robinson *et al.* 1989; Walker *et al.* 1989) and thus allow the use of lower numbers of spermatozoa, but we did not investigate superovulation regimes without GnRH.

Superovulation is known to impair both sperm transport and viability following vaginal or cervical insemination (Evans and Armstrong 1984; Hawk *et al.* 1987), and possibly also after late (Jabbour and Evans 1991) or early intrauterine insemination (Robinson *et al.* 1989). Late insemination may be associated with abnormal fertilization of eggs (Killeen and Moore 1970) or failure of fertilized eggs to cleave (Jabbour and Evans 1991). The inseminations performed at 68 h after pessary removal in the present study may have been too late in some ewes in which ovulations occurred well before the time of semen deposition. This may be particularly important when frozen-thawed semen is used, as the freeze–thawing process also reduces the viability of spermatozoa (Maxwell *et al.* 1990). Moreover, it has been suggested that the utero–tubal junction may act as a barrier to the transport of frozen-thawed spermatozoa if superovulated ewes are inseminated in the uterus after ovulation (Jabbour *et al.* 1987). We were unable to confirm this in our experiments, as all inseminations with frozen-thawed semen were performed at 44 h after progestagen withdrawal.

The results of this study confirm that it is probably important to deposit semen close to, but not coincident with, the median time of ovulation to obtain maximum fertilization

of ova as well as minimizing the loss of ova from surgical manipulation, particularly when oviducal insemination is used. We have also demonstrated the importance of deposition of semen as close as possible to the site of fertilization when numbers of spermatozoa are limiting, as exemplified by the differences in ewe fertility and fertilization of ova between intrauterine and oviducal inseminations.

Robinson *et al.* (1989) found that intrauterine insemination reduced the recovery rate in comparison with cervical insemination, but they obtained poor rates of ova fertilization from the latter insemination method. Our lower recovery rates from oviducal compared with intrauterine inseminations in Experiment 2 suggest that the general handling of the reproductive tract and stress to the ewe associated with the laparotomy procedure interfered with normal ova capture and transport of ova by the oviduct. This is attributable to the surgical procedure rather than the anaesthetic, as the same anaesthetic was used for both types of insemination.

Acknowledgments

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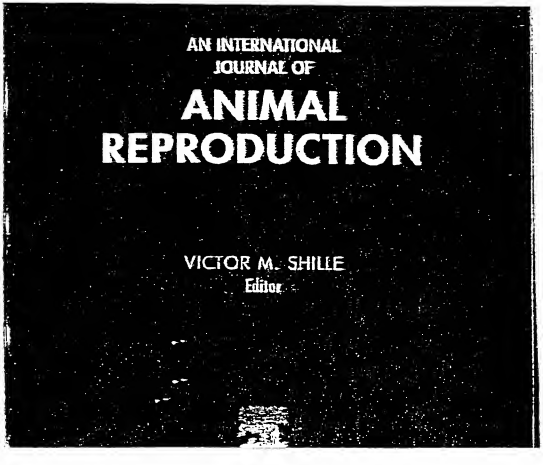
MAASTRICHT, THE NETHERLANDS, JANUARY 9-11, 2000

Naida M. Loskutoff and Brad Stroud,
Program Chairmen

THERIOGENOLOGY



PROCEEDINGS

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SUCCESSFUL LOW-DOSE INSEMINATION BY A FIBEROPTIC ENDOSCOPE TECHNIQUE IN THE SOW

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A large volume insemination dose containing a large number of spermatozoa (2,000-3,000 million) is recommended to achieve good fertility results in sows. However, it is known that only 10 million spermatozoa are sufficient to obtain acceptable pregnancy rates when semen is introduced surgically into the uterotubal junction. Recently, we have developed a fiberoptic endoscope technique for deep uterine horn insemination, as close as possible to the uterotubal junction, without section of the female. With this technique, it is possible to pass the cervical canal and reach the depth of one uterine horn in 3-7 min in about 90% of the sows.

The objective of this study was to determine the effectiveness of inseminating sows with low numbers of spermatozoa using a fiberoptic endoscope technique under field conditions. Three sexually-mature hybrid boars of proven fertility and with satisfactory semen characteristics in the 10 wk preceding the experiment were selected as semen donors. Immediately after collection, sperm-rich fractions of ejaculates were diluted to 1,000 million spermatozoa/mL in BTS diluent. The sperm suspensions from the three boars were mixed and resuspended in BTS diluent to give insemination doses of 1,000, 200 or 50 million mixed sperm cells in 5 mL of diluent. Forty-six multiparous, commercial crossbred sows (parity 3-6) were used in the experiment. Twenty-four hours after weaning (approximately 3 to 4 wk after farrowing), all sows were injected with 1,250 IU eCG (Folligonan: Intervet International B.V., Boxmeer, The Netherlands) for stimulation of follicular growth. Ovulations were induced by an application of 750 IU hCG (Chorulon: Intervet International B.V., Boxmeer, The Netherlands) 72 h after the eCG injection. The animals were inseminated 36 h after hCG injection. Inseminations were performed using a modified artificial insemination spirette to produce a cervical lock and to assist in manipulating a specially designed flexible fiberscope (working length 1.55 m, outer diameter 3.3 mm, instrument channel 1.2 mm) which was inserted through the spirette and moved through the cervical canal into the uterine horn. Silicon spray was used to lubricate the spiral end of the spirette and the endoscope. If after entering one uterine horn we found difficulty in visualizing the entry of the other horn, unilateral insemination was performed. Semen samples (5 mL) were infused into one uterine horn by a syringe attached to the instrument channel of the fiberoptic endoscope. Then, 5 mL of BTS diluent was used to flush the remaining sperm suspension out of the fiberoptic endoscope. Sows were scanned with a 5 MHz sector scanner 24–28 d after insemination to determine the pregnancy rates and were managed until parturition to determine the litter size. Data were evaluated by chi-square test and ANOVA.

Pregnancy rates were 13/15 (86.5%), 16/18 (88.9%), and 12/13 (92.3%) for 1,000, 200 or 50 million sperm per insemination, respectively. Average litter sizes (mean ± SEM) were 9.61 ± 0.29, 9.75 ± 0.31 and 9.41 ± 0.38, respectively. Differences in pregnant rates and litter sizes were not significant. Further experiments are underway to determine critical sperm numbers to maintain a reasonable fertility level for biotechnologies such as sperm sorting where the number of viable spermatozoa is reduced.

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Hysteroscopic insemination of mares with nonfrozen low-dose unsexed or sex-sorted spermatozoa

by

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Summary

Mares were inseminated with low numbers of unsexed and sex-sorted spermatozoa to compare pregnancy rates when using hysteroscopic insemination and deep intrauterine insemination. Semen was collected with an artificial vagina from 2 stallions of known acceptable fertility. Estrus was synchronized (June-July) in 40 mares, ages 3-10, by administering 10mL altrenogest orally for 10 consecutive days, followed by 250 µg cloprostenol i.m. on day 11. All mares were given 3000 iu hCG i.v. at the time of insemination to induce ovulation. Mares were randomly assigned to 1 of 3 treatment groups. Group 1 mares (n=10) were inseminated with 5 x 10^6 spermatozoa deposited deep into the uterine horn with the aid of ultrasonography. Group 2 mares (n=10) were inseminated with 5 x 10^6 spermatozoa deposited onto the utero-tubal junction papillus via hysteroscopic insemination. Group 3 mares (n=20) were inseminated using the hysteroscopic technique with 5 x 10^6 sexed spermatozoa. Spermatozoa were stained with Hoechst 33342 and sorted into X and Y chromosome-bearing populations based on DNA content using an SX MoFlo® sperm sorter. Pregnancy was determined ultrasonographically at 16 days post ovulation. Hysteroscopic insemination resulted in more pregnancies (5/10 = 50%) than did the ultrasound-guided technique (0/10 = 0%; $P<0.05$) when non-sorted sperm were inseminated. Pregnancy rates were not significantly lower ($P>0.05$) when hysteroscopic insemination was used for sorted (5/20 = 25%) and non-sorted spermatozoa (5/10 = 50%). Thus, use of sex-sorted stallion spermatozoa at low numbers in combination with hysteroscopic insemination is capable of fertilization and producing pregnancies of the predetermined sex.

Introduction

High-speed sperm sorting using flow cytometry has been used successfully to produce normal offspring in horses (Buchanan *et al.*, 2000; Schmid *et al.*, 1998), cattle (Seidel *et al.*,

1998), sheep (Cran *et al.*, 1997), rabbits (Johnson *et al.*, 1989), humans (Fugger, 1999), as well as in several other species, and new methods are being developed to enhance the application of this new technology.

The most limiting factor in optimizing the success of artificial insemination with sex-selected semen is the low number of spermatozoa available after sorting. The minimal recommended dose for conventional artificial insemination in the mare is 500 x 10^6 progressively motile sperm (Pickett *et al.*, 1989). Due to the currently low sort rate of around 700 spermatozoa per second, it would take several days to obtain the recommended dose of spermatozoa for artificial insemination. This is not only impractical, but the viability of the spermatozoa would also be significantly reduced. Therefore, low-dose insemination techniques must be developed to reduce the number of spermatozoa needed to maximize fertility (Buchanan *et al.*, 2000; Morris *et al.*, 2000).

The use of the videoendoscope as part of the clinical examination of the mare's reproductive tract (Bracher and Allen, 1992) has enabled a relatively simple, rapid and atraumatic procedure to be developed for the deposition of low numbers of spermatozoa directly onto the papilla of the uterotubal junction (Morris *et al.*, 2000). Hysteroscopic insemination of as few as 5 million spermatozoa onto the papilla of the uterotubal junction resulted in a pregnancy rate of 75%, which is similar to that obtained with conventional intrauterine artificial insemination (Morris *et al.*, 2000). This dose is only 1/100th of that used for conventional uterine insemination, and represents a sufficiently small number of spermatozoa that can be sorted into X and Y chromosome bearing fractions in a reasonable time frame. At current sorting rates of 2.5 million cells per instrument (MoFlo®) per hour, spermatozoa could easily be sorted within 4 hours for hysteroscopic insemination. A similar insemination method for low

numbers of sex-sorted ram spermatozoa has been used to produce successful pregnancies in ewes (Cran *et al.*, 1997).

The two objectives of this study were: 1) to compare the pregnancy rates after insemination of 5 x 10^6 spermatozoa deep into the uterine horn with the aid of ultrasonography (Buchanan et al., 2000), or after deposition of the spermatozoa onto the papilla of the utero-tubal junction using hysteroscopy, and 2) to compare the pregnancy rates of mares inseminated with either 5 x 10^6 non-sorted spermatozoa or 5 x 10^6 sex-sorted spermatozoa.

Materials and Methods

Two Arabian stallions (4 and 6 years old) of proven fertility were used in this experiment. Semen was collected using an artificial vagina (CSU, Animal Reproduction System, Chino, CA) with an in-line gel filter, from each stallion on alternate days throughout the duration of the project. After collection, the semen was evaluated for gel-free volume, motility, and sperm concentration. Samples were extended at a ratio of 10:1 (extender: semen, v/v) with pre-warmed HBGM-3 (Parrish *et al.*, 1988) and centrifuged immediately at room temperature for 15 minutes at 400 x g to concentrate the spermatozoa. After centrifugation, the supernatent containing 90% of the seminal plasma was removed, leaving a soft sperm pellet for further processing.

The oestrous cycles of thirty-four light horse type mares in good body condition and ranging from 3 to 10 years of age, were synchronized by administering a synthetic progestagen altrenogest (0.044mg/kg p.o., Regumate, Hoechst Roussel Vet., Warren, New Jersey, USA) daily for 10 consecutive days. Luteolysis was induced with the prostaglandin analogue, cloprostenol (250µg Estrumate, i.m.; Bayer Corporation, Agriculture Division, Shawnee Mission, Kansas, USA) administered on the eleventh day. The mares' ovaries were examined ultrasonographically every second day until a follicle ≥ 30mm diameter was detected. Then the mares with large

follicles were examined each morning until a follicle ≥ 35mm x 35mm was detected. These mares were randomly assigned to one of the following three treatment groups and inseminated the same afternoon.

Preparation of the inseminate was the same for treatment Groups A and B. After centrifugation, the semen was diluted to provide 100 x 10^6 spermatozoa/ml in a commercial skim milk extender (EZ-Mixin CST®, Animal Reproduction Systems, Chino, CA). The sperm suspension was protected from light and maintained for 6 hours at room (20 - 25°c) temperature to simulate the time needed to sort the spermatozoa for treatment group C. The sperm suspension was then centrifuged through a 45:90% Percoll (Sigma Chemical Co., St. Louis, MO, USA) discontinuous density gradient to reconcentrate the cells and to select a highly motile fraction of spermatozoa. The 90% Percoll was diluted at a ratio of 1:1 (v/v) with HEPES-buffered Tyrode's medium (Grøndahl *et al.*, 1996) to make a 45% solution. In a 15-mL centrifuge tube, 1 mL of 45% Percoll was carefully layered on top of 1 mL of 90% Percoll, then 1 mL of the sperm suspension (100 x 10^6 sperm/mL in EZ-Mixin, CST) was layered on top of the Percoll layers, and the tube was centrifuged at 800 x g for 12 minutes. After centrifugation, the supernatent was completely removed and the pellet was resuspended in 600µl HEPES-buffered Tyrode's Medium. The sperm concentration was determined using a Densimeter (534B MOD-1, Animal Reproduction Systems, Chino, CA) and the required volume to deliver 5 million motile spermatozoa (~100µl) was calculated and prepared for insemination.

Treatment Group A – Ten mares were inseminated with 5 million fresh, non-sorted spermatozoa using an ultrasound-guided technique (Buchanan *et al.*, 2000). The inseminate was deposited at the cranial tip of the uterine horn ipsilateral to the preovulatory follicle using a disposable implant gun designed for use with 0.5-mL straws (Veterinary Concepts, Green Valley, WI). The

location of the implant gun within the uterus was confirmed by transrectal ultrasonography prior to sperm deposition (Buchanan *et al.*, 2000).

Treatment Group B – Ten mares were inseminated with 5 million fresh, non-sorted sperm using the hysteroscopic insemination technique (Morris *et al.*, 2000). The inseminate was prepared by the same method used for treatment Group A. The predetermined dose was then aspirated into an equine GIFT catheter (Cook Veterinary Products, Brisbane, Australia) using a 6-mL disposable syringe attached to the injection port on the distal end of the catheter. The loaded catheter was withdrawn into an outer polypropylene cannula, which was then passed down the working channel of a Pentax EPM 3000 videoendoscope (Pentax UK Ltd, Slough, Bucks UK). The flexible endoscope (1.6m long with an outer diameter of 12 mm) was guided through the cervix and propelled forward through the uterine lumen of the mare (Bracher and Allen, 1992). Under visual control the endoscope was directed along the uterine horn ipsilateral to the ovary containing the preovulatory follicle. When the tip of the endoscope came to within 3-5cm of the papilla of the utero-tubal junction, the outer cannula, and then the inner GIFT catheter containing the sperm suspension, were extruded from the working channel of the endoscope until the tip of the GIFT catheter touched the papilla. The plunger of the syringe was then depressed to deposit the small volume (~100µl) of the inseminate onto the surface of the papilla (Figure1). The endoscope was steadily withdrawn from the uterus while simultaneously evacuating the filtered air that had previously been introduced to facilitate passage of the instrument through the uterine lumen.

Treatment Group C - Twenty mares were inseminated with fresh sex-sorted spermatozoa using the hysteroscopic technique described for Treatment Group B. The concentration of the spermatozoa after centrifugation was determined using the Densimeter®, and a volume of

HBGM-3 was added to provide a sperm concentration of 400 x 10^6 spermatozoa/mL. One mL of the sperm suspension was stained with 25µl Hoescht 33342 (5 mg/mL DDH_2O) and incubated for 1 hour at 34°C. The stained samples were then diluted to 100 x 10^6 sperm/mL for sorting by the addition of 3 mL HBGM-3 containing food coloring (2µl/mL of 1% FD&C #40). The samples were then filtered through a 40 µm filter into 6-mL polypropylene tubes and held at room temperature until further use (Johnson, 1997). Argon lasers, emitting 150 mW at wavelengths of 351 and 364 nm, were used on each of two Cytomation MoFlo (Cytomation, Inc., Fort Collins, CO, USA) flow cytometer/cell sorters modified for sperm sorting at 50 psi. The sheath fluid was HBGM-3 without BSA. Spermatozoa were sorted at approximately 700 live spermatozoa/sec into 50-mL centrifuge tubes. For 14 mares, the sex-sorted spermatozoa were collected into tubes containing 4 mL of a commercial skim milk extender (EX-Mixin CST®) as catch fluid. The remaining 6 mares were inseminated with spermatozoa collected into tubes containing 4 mL of skim milk and egg yolk extender (Squires *et al.*, 1999). Tubes of X-bearing spermatozoa and Y-bearing spermatozoa were pooled separately from each flow cytometer, and centrifuged for 20 minutes at 850 x g at 22°C. The supernatent was aspirated to leave a 200µl sperm pellet. The pellet was then resuspended in 100µl HEPES-buffered Tyrode's medium containing 6% BSA. The final sperm concentration was calculated after counting the spermatozoa using a hemacytometer. The samples were then diluted to a final concentration of 50 x 10^6 spermatozoa/mL in HEPES-buffered Tyrode's medium containing 6% BSA. The predetermined volume (100µl) was then loaded into an equine GIFT catheter and inseminated using the same hysteroscopic technique described for treatment group B.

Ovulation was induced in all mares by the administration of 3000 i.u. human Chorionic Gonadotropin (hCG, Chorulon, Intervet, Inc., Millsboro, Holland), administered intravenously at

the time of insemination or up to a maximum of 8 hours previously. Each mare was inseminated only once, on the side ipsilateral to the ovary containing the dominant follicle. After insemination, mares were examined daily by ultrasound until the day of ovulation. Pregnancy examinations were determined ultrasonographically on days 12, 14, 16, 25, and 35 after ovulation (day of ovulation=0). Pregnancy status was determined using Day 16 examination.

Differences in pregnancy rates were detected using Chi-square analysis of the results. The differences were regarded as significant if $p < 0.05$.

Results

There were no significant differences between the stallions in the final pregnancy rates (Stallion A = 6/19, 31.5%; Stallion B = 4/21, 19%) and so the results were combined from the two stallions. Mares inseminated with the aid of the videoendoscope had significantly higher pregnancy rates than those inseminated at the cranial tip of the uterine horn using the ultrasound guided technique (Table 1).

None of the mares inseminated using the ultrasound guided technique became pregnant compared with 5 out of 10 mares inseminated with non-sorted spermatozoa using the videoendoscope. Pregnancy rates were similar for mares inseminated with fresh non-sorted sperm (50%) and sex-sorted sperm (25%) after insemination using the videoendoscope.

When the pregnancy rates for the two non-sorted treatment groups (A and B) were combined, they were equivalent to those obtained with the sorted spermatozoa. The pregnancy rates were significantly higher in the mares inseminated with the hysteroscopic technique (10/30 mares) compared with no pregnancies obtained with the ultrasound guided technique.

One mare inseminated with Y-bearing sperm lost her pregnancy by 35 days after ovulation and therefore the sex of the fetus could not be determined. One mare inseminated with X-bearing sperm was euthanized 18 days after ovulation due to a gastro-intestinal problem. The conceptus was flushed prior to euthanasia of the mare, and PCR analysis (Peippo *et al.*, 1995) revealed it as a female, the expected sex.

Discussion

Based on the results of this experiment, hysteroscopic insemination directly onto the papilla of the uterotubal junction is the preferred method for insemination of mares, with not only low numbers of spermatozoa (Morris *et al.*, 2000), but also with low numbers of sex-sorted stallion spermatozoa. Normal pregnancies were established from 10 inseminations (30%) using only 5×10^6 sorted or 5×10^6 non-sorted spermatozoa (50%). On the other hand, no pregnancies were obtained after insemination with the ultrasound guided technique (Buchanan *et al.*, 2000) using similarly low numbers of non-sorted sperm. The results of the ultrasound guided deep intrauterine insemination technique differ from those obtained originally by Buchanan *et al.* (2000), who achieved a 35% pregnancy rate using 5×10^6 non-sorted spermatozoa. The reason for this difference is unclear, but it may be due to the additional sperm processing through the Percoll gradient and the lower volume of the inseminate used in the present experiment, or it may be due to the use of different stallions and/or technicians. It is speculated that the low volume (~ 100µl) of the inseminate used for the hysteroscopic insemination has a beneficial effect of maintaining the spermatozoa on the uterotubal junction. However, for deep insemination, a higher volume (~ 500µl) may be required to facilitate passage of the spermatozoa to the site of fertilization in the oviduct.

There appeared to be several advantages to using the hysteroscopic insemination technique. Firstly, the inseminators were able to more precisely deposit sperm directly onto the uterotubal papilla, thus minimizing the loss of the inseminate into the endometrial folds and deep crypts found in the uterus of the estrous mare. In contrast, when the deep intrauterine technique (Buchanan *et al.*, 2000) was used, the inseminators could not be sure of the precise location of sperm deposition. Even though the location of the tip of the pipette was observed ultrasonographically, this provided only a rough estimation of the exact location of the pipette at the time of insemination, since the uterotubal junction could not be visualized by ultrasound. In addition, the location of the uterotubal junction was observed to be quite variable during hysteroscopy, which might decrease the precision of semen deposition using the ultrasound-guided technique.

Furthermore, while guiding the endoscope through the lumen of the mare's uterus, the inseminators could minimize damage to the uterine wall. However, in the ultrasound-guided technique (Buchanan *et al.*, 2000), passage of the pipette through the lumen of the uterus relied solely upon manipulation of the pipette and the uterine horn per rectum, and irritation and damage to the endometrial wall may have occurred. This damage may create an inflamed uterine environment, which would be detrimental to both the sperm viability and subsequent fertilization and embryonic development.

Nevertheless, potential problems may still arise during the hysteroscopic procedure. For example, the endoscope can easily become twisted during passage in the uterus of the mare, resulting in a disorientated video image, and the spermatozoa may be accidentally deposited in the uterine horn contralateral to impending ovulation. Indeed, in this study, one mare was excluded after the scope was determined per rectal palpation to be in the contralateral uterine horn. From this experience, we believe that it is necessary to verify the location of the scope within the uterus, per rectum, prior to sperm deposition.

Furthermore, additional care must be taken to avoid post-insemination endometritis when utilizing the videoendoscope. In our experiment, only one mare was found to have uterine

inflammation following insemination. The low incidence of endometritis observed in this study was due to strict attention to vulval hygiene, cleaning of the endoscope between mares, the use of a very small volume of semen, and insemination of the mares prior to ovulation. It is also necessary to remove the air from the lumen of the uterus immediately after insemination to reduce the irritating effects that air may have on the endometrium (Caslick, 1937). In some cases, it was considered advantageous to gently massage the uterus per rectum to expel the excess air through the cervix of the mare.

There were no significant differences in the pregnancy rates of mares inseminated with non-sorted and sex-sorted sperm in either the present study or the original ultrasound guided insemination technique study (Buchanan *et al.*, 2000). Recent studies in cattle with larger group numbers have shown similar pregnancy rates with both sorted and non-sorted sperm (Seidel *et al.*, 1998). When the results were combined from all previous trials including the insemination of heifers with either sexed or non-sorted control spermatozoa, the pregnancy rates obtained after insemination of sexed sperm were within 90% of the non-sorted controls (Johnson and Welch, 1999).

Buchanan *et al.* (2000) revealed a trend toward higher early embryonic loss rates in mares inseminated with sex-sorted sperm than in the control group. In the present study, 1 of 5 mares (20%) inseminated with sex-sorted spermatozoa lost her pregnancy at 24 days after ovulation. This mare developed an embryonic vesicle that had a normal appearance at Day 16, however, on Day 22, an abnormally small conceptus was detected which contained a fetus, but no heartbeat. By Day 24, no vesicle was present. When using fresh, non-sorted semen, normal early embryonic loss rates have been reported to be 9% by Day 14 and as high as 16% between Days 20 and 50 (Squires, 1998). Therefore, embryonic loss in this study could be considered normal. Similarly, embryonic death rates in cattle have not been increased after insemination with sex-sorted spermatozoa (Seidel *et al.*, 1998). When mares were inseminated with sex-sorted spermatozoa using the ultrasound-guided technique, three of the eight mares (38%) lost their pregnancies between 16 and 60 days after ovulation (Buchanan *et al.*, 2000). However, it is

unlikely that this increase in embryonic loss was due to the sorting process, as it was not repeated in the present study. Rather, the loss may have been due to inflammatory changes associated with endometrial damage incurred during the deep intrauterine insemination procedure. Further studies involving higher numbers of mares inseminated with sex-sorted sperm are needed to determine if embryonic loss will be greater in these mares than in those inseminated with non-sorted sperm.

Another factor that needs further investigation in order to increase the efficacy of inseminating mares with sex-sorted semen is determination of the appropriate number of sorted spermatozoa required for satisfactory fertility (Buchanan *et al.*, 2000, Morris *et al.*, 2000). The ideal insemination dose for sex-sorted stallion spermatozoa will involve the lowest possible number of spermatozoa that can be used routinely to produce fertility rates within 90% of those rates resulting from conventional artificial insemination. Current pregnancy rates from insemination with sex-sorted spermatozoa in the horse at 16 and 60 days after ovulation are, respectively, 25% and 20% when using 5 x 10^6 motile cells, and 40% and 25% when using 25 x 10^6 total cells (Buchanan *et al.*, 2000). Since these experiments utilized different insemination techniques, a comparison between the two studies is not justified. In a recent study using hysteroscopic insemination technique, Morris *et al.* (2000) reported that insemination of only 1 x 10^6 Percoll-treated non-sorted spermatozoa at the uterotubal junction resulted in satisfactory pregnancy rates (64%), which were equivalent to those obtained by conventional intrauterine insemination. It is possible that a similar insemination dose could be used successfully with sex-sorted semen, but further experiments are necessary to determine the minimal sperm number.

In this study, the inseminations were performed some 30 - 36 h prior to the anticipated time of ovulation. Further investigation to determine the optimum time of insemination using sex-sorted spermatozoa is required. A major difference between the control hysteroscopic insemination group and the sex-sorted treatment group was that the sex-sorted spermatozoa had been processed over several hours through the flow cytometer, compared with the control semen which was maintained in a skim-milk based extender for an equivalent time period and then

centrifuged for 12 minutes through the Percoll density gradient. It may be that insemination of the sex-sorted semen at a time close to ovulation to compensate for reduced sperm longevity would improve the pregnancy rates.

In summary, hysteroscopic insemination is a practical technique for insemination of low numbers of sex-sorted stallion spermatozoa. Hysteroscopic insemination at the uterotubal junction is a relatively non-invasive and straightforward procedure which can be undertaken in the majority of mares, and thus could be easily incorporated into many modern breeding centers. The use of this technology, coupled with high-speed flow-cytometric sorting (Johnson *et al.*, 1989) in this trial, has produced 3 apparently normal pregnancies of the predetermined sex using only 5 x 10^6 motile spermatozoa. With continuing advances being made in these research areas, it is conceivable that this technology could be made available to commercial artificial insemination programs in the near future.

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338 Table 1.
339 Pregnancy rates for mares inseminated with unsorted and sex-selected spermatozoa.
340

Treatment	Number of pregnant mares out of those inseminated	Percentage of pregnant mares at 16 days after ovulation
Ultrasound, Non-sorted	0/10	0[a]
Hysteroscopy, Non-sorted	5/10	50.0[b]
Hysteroscopy, Sex-sorted	5/20	25.0[a,b]
Skimmilk	4/14	28.5
Modified Skimmilk + Egg Yolk	1/6	16.6

341 [a,b] Values with different superscripts differ ($P<0.05$).

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Authors should give sufficient details of the experimental design and analysis so that the reader can assess their adequacy and validity for testing the hypotheses of interest. In particular, the numbers of experimental units used and the way in which they have been allocated to treatments should be described. If observations have been omitted from the analysis this must be justified. Methods of analysis should be described precisely and any necessary assumptions stated clearly because these may affect the conclusions that can be drawn from the experiment.

Results

Results should be presented in text, tables and figures as appropriate. Data given in tables and figures should not be repeated in the text. Tables and figures should be cited in the text in numerical order.

Discussion

The interpretation of results should be discussed, observations should be related to relevant studies and the implications of results for future research should be outlined. Results should not be repeated in this section. The discussion should not be more than 15% of the total length of the paper.

Acknowledgement

Acknowledgement of technical help and of financial and material support may be mentioned in this section.

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Please ensure that all references cited in the text are included in the reference list and vice versa. The reference list should contain only articles that are accessible to most scientists. Unpublished work, including personal communications, manuscripts in preparation and manuscripts submitted but not yet accepted for publication, should be referred to in the text in the following way: (A. Stone, unpublished) (J. Brown, personal communication). Articles that are accepted for publication but are not yet published may be listed as 'in press' in the reference list.

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(Davies and Smith, 1996; Frost *et al.*, 1997; Williams, 1997).

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Newton H, Pegg DE, Barrass R and Gosden RG (1999) Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulphoxide of human mature oocytes *Journal of Reproduction and Fertility* 117 27–33

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Byskov AG and Hoyer PE (1994) Embryology of mammalian gonads and ducts. In *The Physiology of Reproduction* pp 75–56 Eds E Knobil and JD Neill. Raven Press, New York

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Tables should be concise and informative. The title should be a single sentence at the head of the table and should include the name of the organism studied. Any additional explanatory material should appear as footnotes cross-referenced to the column entries. Tables should be self-contained, ie, not requiring further explanation; they should be numbered (arabic numbers) and cited in the text. All abbreviations used in the table must be explained in the footnotes. Each column should have a short heading. Internal horizontal and vertical rules should not be used.

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Abbreviations that can be used without definition:

ANOVA	BSA	cAMP	DNase
eCG	EDTA	EGTA	ELISA
FSH	GnRH	hCG	Hepes
HPLC	IgG	IVF	LH
PBS	RT–PCR	SDS	Tris

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Low dose insemination of mares using non-sorted and sex-sorted sperm

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Abstract

Mares are generally inseminated with 500 million progressively motile fresh sperm and approximately 1 billion total sperms that have been cooled or frozen. Development of techniques for low dose insemination would allow one to increase the number of mares that could be bred, utilize stallions with poor semen quality, extend the use of frozen semen, breed mares with sexed semen and perhaps reduce the incidence of post-breeding endometritis. Three low dose insemination techniques that have been reported include: surgical oviductal insemination, deep uterine insemination and hysteroscopic insemination.

Insemination techniques: McCue et al. [J. Reprod. Fert. 56 (Suppl.) (2000) 499] reported a 21% pregnancy rate for mares inseminated with 50,000 sperms into the fimbria of the oviduct.

Two methods have been reported for deep uterine insemination. In the study of Buchanan et al. [Theriogenology 53 (2000) 1333], a flexible catheter was inserted into the uterine horn ipsilateral to the corpus luteum. The position of the catheter was verified by ultrasound. Insemination of 25 million or 5 million spermatozoa resulted in pregnancy rates of 53 and 35%, respectively. Rigby et al. [Proceedings of 3rd International Symposium on Stallion Reproduction (2001) 49] reported a pregnancy rate of 50% with deep uterine insemination. In their experiment, the flexible catheter was guided into position by rectal manipulation.

More studies have reported the results of using hysteroscopic insemination. With this technique, a low number of spermatozoa are placed into or on the uterotubal junction. Manning et al. [Proc. Ann. Mtg. Soc. Theriogenol. (1998) 84] reported a 22% pregnancy rate when 1 million spermatozoa were inserted into the oviduct via the uterotubal junction. Vazquez et al. [Proc. Ann. Mtg. Soc. Theriogenol. (1998) 82] reported a 33% pregnancy rate when 3.8 million spermatozoa were placed on the uterotubal junction. Recently, Morris et al. [J. Reprod. Fert. 188 (2000) 95] utilized the hysteroscopic insemination technique to deposit various numbers of spermatozoa on the uterotubal junction. They reported pregnancy rates of 29, 64, 75 and 60% when 0.5, 1, 5 and 10 million spermatozoa, respectively, were placed on the uterotubal junction.

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Insemination of sex-sorted spermatozoa: One of the major reasons for low dose insemination is insemination of X- or Y-chromosome-bearing sperm. Through the use of flow cytometry, spermatozoa can be accurately separated into X- or Y-bearing chromosomes. Unfortunately, only 15 million sperms can be sorted per hour. At that rate, it would take several days to sort an insemination dose containing 800 million to 1 billion spermatozoa. Thus, low dose insemination is essential for utilization of sexed sperm.

Lindsey [Hysteroscopic insemination with low numbers of fresh and cryopreserved flow-sorted stallion spermatozoa, M.S. Thesis, Colorado State University, Fort Collins, CO, USA, 2000] utilized either deep uterine insemination or hysteroscopic insemination to compare pregnancy rates of mares inseminated with sorted, fresh stallion sperm to those inseminated with non-sorted, fresh stallion sperm. Hysteroscopic insemination resulted in more pregnancies than ultrasound-guided deep uterine insemination. Pregnancy rate was similar for mares bred with either non-sorted or sex-sorted spermatozoa.

In a subsequent study, Lindsey et al. [Proceedings of 5th International Symposium on Equine Embryo Transfer (2000) 13] determined if insemination of flow-sorted spermatozoa adversely affected pregnancy rates and whether freezing sex-sorted spermatozoa would result in pregnancies. Mares were assigned to one of four groups: group 1 was inseminated with 5 million non-sorted sperms using hysteroscopic insemination; group 2 was inseminated with 5 million sex-sorted sperms using hysteroscopic insemination; group 3 was inseminated with non-sorted, frozen-thawed sperm; and group 4 was inseminated with sex-sorted frozen sperm. Pregnancy rates were similar for mares inseminated with non-sorted fresh sperm, sex-sorted fresh sperm and non-sorted frozen sperm (40, 37.5 and 37.5%, respectively). Pregnancy rates were reduced dramatically for those inseminated with sex-sorted, frozen-thawed sperm (2 out of 15, 13%). These studies demonstrated that hysteroscopic insemination is a practical and useful technique for obtaining pregnancies with low numbers of fresh spermatozoa or low numbers of frozen-thawed spermatozoa. Further studies are needed to determine if this technique can be used to obtain pregnancies from stallions with poor semen quality. In addition, further studies are needed to develop techniques of freezing sex-sorted spermatozoa.

Keywords: Sexed; Stallion; Sperm; Low dose; Artificial insemination (AI)

1. Introduction

Generally, mares are inseminated with 500 million progressively motile fresh spermatozoa, approximately 1 billion total sperms that have been cooled, and 800 million to 1 billion spermatozoa that have been frozen and thawed (Squires et al., 1999). This large sperm number certainly limits the number of mares that can be inseminated with a single ejaculate. The uterus serves as a filter device that allows only a small percentage of the spermatozoa to reach the oviduct (Rigby et al., 2000). Of these spermatozoa, a very high percentage are morphologically normal. The majority of mares inseminated experience a post-breeding endometritis which is generally resolved by 24 h post-breeding. Some older mares may exhibit more severe post-breeding endometritis and accumulate fluid (Troedsson, 1999). The time required to resolve the post-breeding endometritis appears to be slightly longer for mares inseminated with frozen-thawed spermatozoa (Troedsson et al., 1998).

One possible means of diminishing the post-breeding endometritis would be to deposit the sperm directly on the uterotubal papilla at the anterior tip of the uterine horn ipsilateral

to the ovary containing the dominant preovulatory follicle. Insemination directly on the uterotubal junction may also be beneficial for situations where: frozen semen is in limited supply, semen from a subfertile stallion is being used, stallions are heavily booked and the number of spermatozoa are limited, and for insemination of sex-sorted sperm.

Due to the current rates of sorting spermatozoa into X- and Y-chromosome-bearing populations, minimal numbers of sex-sorted spermatozoa are available for insemination. Deep uterine insemination is a proven technique in cattle that allows sperm numbers to be decreased without dramatically affecting fertility. Pregnancy rates after insemination of sex-sorted sperm currently are within 70 to 90% for heifers inseminated with non-sorted sperm (Seidel et al., 1999b). A method for sorting X- and Y-chromosome-bearing spermatozoa has been developed in the horse, but only limited trials have been conducted to determine the fertility of sex-sorted sperm. The first reported pregnancy from flow-sorted stallion spermatozoa was produced by surgical oviductal insemination (Schmid et al., 2000). Subsequently, Buchanan et al. (2000) reported a 40% pregnancy rate when 25 million flow-sorted spermatozoa were deposited deep into the uterine horn.

Development of techniques for insemination of low numbers of both sex-sorted and non-sorted frozen-thawed stallion spermatozoa is of major interest. This is particularly true for stallions with a limited inventory of frozen semen, for stallions that are dead, or no longer capable of producing fertile sperm, and for those stallions that have poor semen quality after cryopreservation. This report summarizes several studies on pregnancy rates of mares inseminated with low numbers of either non-sorted or sex-sorted fresh or frozen-thawed spermatozoa inseminated by deep uterine insemination using a flexible catheter or by use of a videoendoscope.

2. Low-dose, non-sorted sperm

Maximum fertility has been achieved in mares with the insemination of 500 million progressively motile spermatozoa (Pickett and Voss, 1975). Under ideal conditions with certain highly fertile stallions, fertility remained unchanged when the insemination dose was lowered to 100 million progressively motile fresh spermatozoa. However, when the spermatozoal numbers were reduced to 50 million progressively motile fresh spermatozoa, fertility was lowered (Householder et al., 1981).

One method that has been used to achieve acceptable fertility rates with reduced sperm numbers is oviductal insemination. Maxwell et al. (1993), using a laparoscopic method, achieved higher pregnancy rates with oviductal insemination (61%) compared to intrauterine insemination (39%) in sheep. A similar procedure has produced viable pregnancies in humans (Berger, 1987) and swine (Morcom and Dukelow, 1980). When attempted in horses, McCue et al. (2000) found no difference between traditional artificial insemination of 500 million progressively motile spermatozoa placed in the uterine horn versus 50,000 progressively motile spermatozoa deposited via surgical oviductal insemination. Recently, Coutinho da Silva et al. (2001) transferred oocytes into the oviducts of recipient mares and either placed semen in the oviduct simultaneously or inseminated the mares. They found no difference in pregnancy rates between mares in which 200,000 sperms were inseminated into the oviduct versus those in which 500 million spermatozoa were inseminated

into the uterine body. Unfortunately, oviductal insemination is not a practical method of insemination and thus a less invasive method is preferable.

Several studies have examined the possibility of using a videoendoscope to deposit spermatozoa on and around the uterotubal papilla. The uterotubal papilla has been suggested to be an important site for preovulatory sperm storage. In a recent study, Scott et al. (2000) found spermatozoa on the uterine side of the uterotubal junction in various glands and crypts in artificially inseminated mares. Manning et al. (1998) reported a non-surgical method in which the inseminate was deposited through the orifice of the uterotubal papilla with the aid of a videoendoscope. These researchers achieved a pregnancy rate of 22% (2 out of 9) after deposition of 1 million progressively motile spermatozoa. In the same study, no pregnancy was reported ($n = 10$) following insemination of 10 million spermatozoa in the same manner. The poor results in this study were attributed to the difficulty in attempting to cannulate the uterotubal papilla. Vazquez et al. (1998) also used hysteroscopic insemination to deposit 3.8 million progressively motile spermatozoa on the uterotubal papilla and achieved a pregnancy rate of 30% (3 out of 10).

Morris et al. (2000) evaluated the fertility of mares inseminated hysteroscopically with various numbers of progressively motile spermatozoa. They extended spermatozoa with a skimmilk-based extender and layered the extended semen onto a 90:45 Percoll discontinuous density gradient. The sperm suspension was centrifuged through the Percoll layers for 5 min at 200 g and subsequently for 10 min at 800 g. Insemination doses of 10, 5, 1, 0.5, 0.1 or 0.001 million motile spermatozoa were compared. The pre-determined insemination dose of motile spermatozoa, suspended in 30 to 150 μl of Tyrode's medium, was aspirated into an equine GIFT catheter. The loaded catheter was drawn into an outer polypropylene cannula and passed through the working channel of a Pentax EPN 3000 videoendoscope. The videoendoscope was guided through the cervix and propelled forward through the uterine lumen of the estrous mare as described by Bracher and Allen (1992). Under visual control, the endoscope was directed along the uterine horn ipsilateral to the ovary containing the preovulatory follicle. When the tip of the endoscope came to within 3–5 cm of the papilla of the uterotubal junction, first the outer cannula and then the inner GIFT catheter containing the sperm suspension was extruded from the working channel until the tip of the GIFT catheter touched the papilla. The plunger of the syringe was then depressed to deposit the small volume of inseminate onto the papilla. Pregnancy rates for those inseminated with 10, 5 and 1 million spermatozoa were 60, 75 and 64%, respectively. Pregnancy rates decreased dramatically when sperm numbers were less than 1 million progressively motile spermatozoa. These authors concluded that the higher pregnancy rates obtained in their study, compared to those of Manning et al. (1998) and Vazquez et al. (1998), may be attributed not only to the use of Percoll gradient centrifugation to remove seminal plasma, but also to the small volume of inseminate used.

Another method used to achieve pregnancies with suboptimal numbers of spermatozoa is insemination of spermatozoa near the tip of the uterine horn ipsilateral to the ovary containing the dominant follicle. Buchanan et al. (2000) compared pregnancy rates in mares inseminated on a single occasion close to ovulation with 5, 25 or 500 million progressively motile spermatozoa (Table 1). Mares were assigned to one of three treatments. (1) Mares were inseminated with 500 million progressively motile spermatozoa deposited into the uterine body using a flexible plastic AI pipette. (2) Mares were inseminated with 25 million

Table 1
Pregnancy rates resulting from insemination of low numbers of stallion sperm[a]

Progressively motile sperm	Volume of inseminate (ml)	Mares pregnant/ mares inseminated	Pregnancy rates (%)[b]
500×10^6	20	18/20	90a
25×10^6	1	12/21	57b
5×10^6	1	3/10	30b
5×10^6	0.2	4/10	40b

[a] Adapted from Buchanan et al. (2000).
[b] Values with different letters differ ($P < 0.05$; χ^2).

progressively motile spermatozoa in 1 ml of dried skimmilk extender (E-Z Mixin CST®, Animal Reproduction Systems, Chino, CA, USA). Semen was deposited at the tip of the uterine horn ipsilateral to the preovulatory follicle using a flexible plastic AI pipette. Location of the pipette within the uterus was confirmed by transrectal ultrasonography. (3) Mares were inseminated with 5 million progressively motile spermatozoa in either 1 ml of dried skimmilk extender or 0.2 ml of dried skimmilk extender. Mares were inseminated as per treatment 2. More ($P < 0.05$) mares became pregnant when inseminated with 500 million (18 of 29, 90%) than with 25 million (12 of 21, 57%) progressively motile spermatozoa, but pregnancy rates were similar for mares inseminated with 25 million versus 5 million (7 of 20, 35%) progressively motile spermatozoa. In a similar study (Woods et al., 2000), identical sperm numbers (25 million spermatozoa) were deposited into the uterine body or in the uterine horn. Pregnancy rates were not altered by deep uterine horn insemination.

A further study was completed to compare pregnancy rates when sperms were deposited either deep within the uterine horn using the flexible AI pipette or on and around the uterotubal papilla by use of a videoendoscope (Rigby et al., 2001). All inseminations consisted of 5 million sperms from a single, highly fertile stallion that had been cooled and stored for 24 h. No differences in pregnancy rates were found between the pipette technique (10 of 20, 50%) and the videoendoscopic insemination method (13 of 21, 62%; $P > 0.05$).

Conflicting data exist regarding the efficacy of deep intrauterine insemination in cattle. Cornual insemination was shown to be advantageous in heifers by Seidel et al. (1997, 1998). In contrast, McKenna et al. (1990) found no increase in pregnancy rates of dairy cattle when deep intrauterine insemination was used compared to traditional insemination in the uterine body. Additionally, in a review article (Seidel et al., 1999b) that combined insemination trials in heifers, no advantage of horn insemination was detected. One reason for conflicting data in cattle is technician expertise or error. In cattle, horn insemination is more likely to result in intrauterine semen deposition, whereas body insemination may result in at least a portion of the semen being deposited intracervically (Senger et al., 1988). This problem is unlikely to be significant in mares due to the difference in insemination technique. Further studies are needed in the horse to determine if depositing low numbers of spermatozoa deep into the uterine horn is of advantage to deposition into the uterine body. More than likely this will depend upon the inherent fertility of the stallion. Additional studies are required to determine if those stallions in which fertility is not maximal may be aided by deep uterine insemination.

One area of low dose insemination that has received considerable attention in the past year is the possibility of inseminating low numbers of frozen-thawed spermatozoa onto the uterotubal junction. If this procedure would provide satisfactory pregnancy rates, then the numbers of frozen-thawed spermatozoa that are needed for insemination could be decreased dramatically. This is of extreme interest since it is quite costly to collect and freeze stallion spermatozoa. Unfortunately, although there are numerous anecdotal reports of improved pregnancy rates with videoendoscopic insemination of frozen-thawed spermatozoa, there are limited studies available. In a study conducted in our laboratory, pregnancy rates were compared for mares inseminated with 5 million non-sorted fresh sperm versus 5 million non-sorted, frozen-thawed spermatozoa (Lindsey et al., 2000; Squires et al., 2000). Mares were inseminated a single time using a videoendoscopic insemination method. Four out of ten mares (40%) became pregnant when inseminated with non-sorted fresh spermatozoa versus 6 out of 16 mares (38%) inseminated with non-sorted, frozen-thawed spermatozoa. The pregnancy rates were not different between fresh and frozen-thawed spermatozoa. Furthermore, the pregnancy rate obtained with only 5 million frozen-thawed spermatozoa (38%) was similar to that obtained when 800 million to 1 billion total frozen-thawed spermatozoa are inseminated into the uterine body (Squires et al., 1999). Further studies are needed with larger numbers of mares to determine if the total number of frozen-thawed spermatozoa can be decreased dramatically by using videoendoscopic insemination onto the uterotubal junction.

3. Low dose insemination of sex-sorted sperm

The ability to pre-select the sex of offspring has tremendous advantages in livestock species. The advantages of sex pre-selection in the horse are less evident, but still very desirable for most breeders. Johnson et al. (1989) were the first to report a reliable method to predetermine sex using DNA as a quantitative marker for X- and Y-chromosome-bearing spermatozoa and sorting spermatozoa via flow cytometry. The first reported foal produced from insemination of sex-sorted sperm was the result of surgical insemination into the oviduct of 150,000 sperms (Schmid et al., 2000). Subsequently, Buchanan et al. (2000) inseminated mares with sperm that had been sorted into X- and Y-chromosome-bearing populations using flow cytometry (Table 2). Mares from one group were inseminated on a single occasion using approximately 25 million live, sorted sperm in 1 ml of E–Z Mixin® extender (Animal Reproduction Systems, Chino, CA, USA). Sperms were deposited at the tip of the uterine horn ipsilateral to the preovulatory follicle using a

Table 2
Pregnancy rates resulting from insemination of 25 × 10^6 sex-sorted stallion sperm[a]

Treatment	Mares inseminated	Pregnant at 16 days (%)	Pregnant at 60 days (%)
E–Z Mixin®	10	3[b] (30)	1 (10)
E–Z Mixin® + 4% EY	10	5[b] (50)	4 (40)

[a] Adapted from Buchanan et al. (2000).
[b] No significant difference ($P > 0.1$; Fisher's exact test).

flexible plastic AI pipette. The location of the pipette within the uterus was confirmed by transrectal ultrasonography prior to semen deposition. Mares in the second group were inseminated as described for the previous group except that 4% egg yolk was added to the skimmilk extender. Pregnancy rates at 16 days post-ovulation after insemination of sex-sorted spermatozoa were 3 of 10 (33%) for mares inseminated with sorted sperm in E–Z Mixin® extender and 5 of 10 (50%) for those inseminated with sorted sperm in E–Z Mixin® plus 4% egg yolk. Five out of twenty (25%) mares remained pregnant at 60 days of gestation.

During the 1999 breeding season, a study was conducted in our laboratory (Lindsey et al., 2000) to compare the effectiveness of inseminating 5 million spermatozoa into the mare's uterine horn using ultrasound-guided deep uterine insemination or by depositing spermatozoa directly onto the papilla of the uterotubal junction by hysteroscopy. A second objective was to compare the ability of non-sorted sperm and flow-sorted spermatozoa to initiate pregnancy in mares when 5 million sperms were hysteroscopically inseminated. Mares were randomly assigned to one of three treatment groups. (1) Mares were inseminated with 5 million motile spermatozoa deep into the uterine horn with the aid of a flexible pipette and ultrasonography. (2) Mares were inseminated with 5 million motile spermatozoa deposited directly onto the uterotubal papilla using a videoendoscope. (3) Mares were inseminated with 5 million motile flow-sorted spermatozoa using a videoendoscope. The procedure for videoendoscopic insemination was identical to that described by Morris et al. (2000). Pregnancy status was determined at 16 days post-ovulation.

Hysteroscopic insemination resulted in higher numbers of pregnancies (5 of 10, 50%) than did the ultrasound-guided insemination (0 of 10, 0%) when non-sorted sperms were inseminated. However, pregnancy rates were similar for mares that were hysteroscopically inseminated with sorted (5 of 20, 25%) or non-sorted (5 of 10, 50%) spermatozoa. The fertility rate using ultrasound-guided, deep uterine insemination was different from that reported by Buchanan et al. (2000) who achieved a 35% pregnancy rate using 5 million non-sorted sperm. The reason for this difference was unclear, but may be due to the additional processing steps for sperm that occurred in the latter study, or due to the use of different stallions or technicians. The similar pregnancy rates for mares inseminated with non-sorted and flow-sorted stallion spermatozoa in the present study agrees with the results of Buchanan et al. (2000). In cattle (heifers), similar pregnancy rates for both non-sorted and sorted sperms were reported recently from large field trials (Seidel et al., 1999b).

It was concluded from this study that hysteroscopic insemination is a practical technique for insemination of low numbers of flow-sorted stallion spermatozoa. Hysteroscopic insemination of sperm at the uterotubal junction is a relatively non-invasive method that could be easily incorporated into many modern breeding centers.

The ability to cryopreserve flow-sorted spermatozoa would greatly increase the practicality of using sex-sorted sperm in the horse industry. Stallion spermatozoa survive only a short period of time after sorting. Therefore, mares must be inseminated immediately after sperm sorting. However, if sperm could be frozen following sorting, they could be used at a future time or different location. Cryopreservation of flow-sorted bull spermatozoa has been used extensively. Seidel et al. (1999a) reported the pregnancy rates for heifers

Table 3
Pregnancy rates following hysteroscopic insemination of 5×10^6 motile fresh or frozen/thawed sex-sorted stallion sperm

Treatment	Mares inseminated	Mares pregnant	Pregnancy rate (%)[a]
Fresh non-sorted	10	4	40
Fresh sex-sorted	16	6	37.5
Frozen/thawed non-sorted	16	6	37.5
Frozen/thawed sex-sorted	15	2	13.3

[a] No significant difference ($P > 0.05$; χ^2).

inseminated with flow-sorted, frozen-thawed spermatozoa (18 of 35, 51%) to be similar to that for those inseminated with frozen-thawed, non-sorted spermatozoa (27 of 37, 73%). Seidel et al. (1999b) also reported that pregnancy rates for heifers inseminated with low numbers of sorted, frozen-thawed sperms are generally within 90% of rates obtained with non-sorted, frozen-thawed sperm samples containing 7–20 times more spermatozoa per insemination.

A 2 × 2 factorial experiment was conducted to determine the effects of sorted versus non-sorted and fresh versus frozen spermatozoa (Lindsey et al., 2000; Table 3). Mares were assigned to one of four treatments. Treatment 1 consisted of fresh, nonsorted sperm. Treatment 2 consisted of fresh, flow-sorted spermatozoa. Sperms to be sorted were stained with Hoechst 33342 and sorted into X- and Y-chromosome-bearing spermatozoa based on DNA content using an SX Mo-Flo sperm sorter (Lindsey, 2000). Treatment 3 consisted of frozen-thawed, non-sorted sperm. Treatment 4 consisted of flow-sorted, frozen-thawed spermatozoa. Concentrations of sperm in both cryopreserved treatments were adjusted based on pre-determined average post-thaw motilities, so that each insemination consisted of approximately 5 million motile spermatozoa. Hysteroscopic insemination of 5 million motile spermatozoa in a volume of 230 μl was used for all treatments, and pregnancy was determined ultrasonographically 16 days post-ovulation.

There was no difference found in pregnancy rates for mares inseminated with fresh, non-sorted (4 of 10, 40%); fresh, flow-sorted (6 of 16, 37.5%); frozen-thawed, non-sorted (6 of 16, 37.5%) and flow-sorted, frozen-thawed spermatozoa (2 of 15, 13.3%; $P > 0.05$). Pregnancy rates tended ($P = 0.12$) to be lower following insemination of flow-sorted, frozen-thawed spermatozoa. Further studies are needed with a larger number of mares to determine if fertility of flow-sorted, frozen-thawed spermatozoa can be improved. Based on the 13% pregnancy rate obtained in this study for flow-sorted, frozen-thawed spermatozoa, this procedure cannot be recommended at this time.

Currently, in the cattle industry, bulls are sent to a centralized facility that has the equipment and expertise to sort spermatozoa into X- and Y-chromosome-bearing populations and then freeze the sorted sperm. Unfortunately, our study demonstrated that flow-sorted, frozen-thawed equine spermatozoa may provide decreased fertility.

A possible alternative to freezing sorted stallion sperm is to ship fresh semen to a centralized facility, sort the spermatozoa and then inseminate the mares immediately. Shipped semen is used routinely in the equine industry, therefore this may provide a more applicable use of this technology. A recent study was conducted to compare the fertility of sperms that

Table 4
Pregnancy rates following hysteroscopic insemination of 20 × 10^6 fresh or stored sex-sorted stallion sperm

Treatment	Mares inseminated	Mares pregnant	Pregnancy rate (%)[a]
Fresh sex-sorted	20	6	30
18 h stored sex-sorted	20	7	35

[a] No significant difference ($P > 0.05$; χ^2).

were flow-sorted immediately following collection to that of sperms that were stored for 18 hr prior to flow-sorting (Table 4). Treatment 1 consisted of 20 million sperms that had been flow-sorted immediately following collection, using a method similar to that described by Lindsey (2000). Treatment 2 consisted of 20 million sperms that had been stored for 18 hr and then sorted by flow-cytometry. Sperm for treatment 2 were stored at a concentration of 25 million sperm/ml in E–Z Mixin® CST. These samples were stored at 20 °C to avoid future complications that occur in the flow-sorting process when chilled semen is used. After storage, sperms were processed for flow-sorting, similar to the method described previously for stallion spermatozoa (Lindsey, 2000). Pregnancy was determined based on ultrasound examination on day 16 post-ovulation.

The results of this study indicate that storage of sperm prior to flow-sorting does not alter pregnancy rates. Pregnancy rates were similar for those mares inseminated with fresh flow-sorted spermatozoa (6 of 20, 30%) and those inseminated with stored, flow-sorted spermatozoa (7 of 20, 35%; $P > 0.05$). One mare inseminated with stored, flow-sorted spermatozoa ovulated two follicles, and twins were detected upon ultrasound examination. For our results, this was counted as a single pregnancy. Based on the results of this study, semen could be collected on the farm, shipped to a facility for flow sorting and mares inseminated with sexed sperm.

4. Summary

Past and present research has established reproductive technologies which have become an integral part of the global equine industry. Artificial insemination and cryopreservation of stallion spermatozoa are examples of technologies that have become commercially available to horse owners. One recent technology examined is insemination of low numbers of spermatozoa per inseminate. The two simplest and most practical procedures appear to be deep uterine insemination and hysteroscopic insemination. These two methods are capable of producing similar pregnancy rates from the insemination of low numbers of stallion sperm (Rigby et al., 2001). However, in some cases, such as when using highly stressed sperm or flow-sorted sperm, an advantage could likely be gained if the insemination is performed with the aid of a videoendoscope (Lindsey et al., 2000). Other instances in which hysteroscopic insemination could be of great value are for the insemination of low numbers of frozen-thawed sperm; flow-sorted, frozen-thawed; and stored, flow-sorted stallion sperm. With the recent development of these low-dose insemination technologies, many options become available for the use of existing inventories of frozen semen, as well as for the use of flow-sorted stallion spermatozoa.

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PRODUCTION OF LAMBS BY LOW DOSE INTRAUTERINE INSEMINATION WITH FLOW CYTOMETRICALLY SORTED AND UNSORTED SEMEN

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Lambs of predetermined sex have not been produced except for one by ICSI (Catt et al., 13th ICAR 3:24–4, 1996). Low sperm sort rates (up to 1 x 10^6 per h) and sub-optimal timing of insemination relative to ovulation (Maxwell et al., Reprod. Fert. Dev. 5:57-63, 1993) are major limiting factors.

In February 1996, towards the end of seasonal estrus at latitude 57°N, 2 trials were undertaken. Semen from a single Suffolk ram was collected and diluted 1:1 in Zorpva extender until used. Sperm were sorted using a Becton Dickinson FACStar Plus flow cytometer/cell sorter with a forward fluorescence detector, bevelled sample injection needle, and UV laser output of 175 MW. Sperm were sorted at approximately 0.9 x 10^6 per h for X and 0.5 x 10^6 per h for Y into Zorpva containing 20% egg yolk. Sorted sperm, and similar numbers of untreated sperm, were stored at room temperature until used. Ewes were synchronised for estrus by 12 d progestogen priming (30 mg Chronogest; Intervet, UK) followed by 400 IU PMSG (Intervet, UK) administered im at progestogen withdrawal.

In trial 1, 30 Bluefaced Leicester x Scottish Blackface ewes were inseminated between 51.5 and 53.0 h after progestogen withdrawal using sperm from a single semen collection made 2.5 h prior to the start of AI. In the second trial, 60 Scottish Blackface ewes were used; semen was collected twice, 8.5 and 1.5 h before AI commenced; and inseminations carried out between 53.5 and 56.5 h after progestogen withdrawal. All inseminations were by laparoscopic intrauterine AI and approximately 100,000 spermatozoa, sorted or unsorted, in 0.1 ml medium were deposited at the tip of each uterine horn. Care was taken to direct the tip of the glass inseminating pipette towards the utero-tubal junction at semen delivery.

In trial 1, none of the 18 ewes inseminated with X sperm lambed; but 5/12 inseminated with unsorted sperm produced 1 female and 6 male lambs. In trial 2, none of 5 ewes inseminated with Y sperm lambed; 4/25 inseminated with X sperm, and 2/30 inseminated with unsorted sperm lambed. The 4 ewes which received X sperm produced a total of 6 normal female lambs at term. The 2 ewes lambing after AI with unsorted semen each produced a single female lamb. All 6 pregnancies occurred in ewes inseminated towards the end of the insemination period, i.e. 55 to 56 h after progestogen withdrawal. Semen collected 8.5 h before insemination resulted in 2 pregnancies whereas that collected 1.5 h before AI produced 4 pregnancies.

The pregnancy rates achieved were low probably due to a combination of factors including the delay between semen collection and insemination, asynchrony between insemination and ovulation, semen dose, and the onset of seasonal anestrus. These trials, however, have demonstrated, for the first time, that pregnancy in sheep can be established and lambs of predetermined sex obtained by the deposition of a low dose of sorted semen close to the utero-tubal junction of the ewe.

Sexing / Sex Ratio

PREGNANCY RATES IN MARES FOLLOWING A SINGLE INSEMINATION WITH A LOW NUMBER OF SPERMATOZOA INTO THE TIP OF THE UTERINE HORN

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Insemination with a low number of spermatozoa is necessary when semen is limited or when using sorted sexed semen. Therefore, a study was done to compare pregnancy rates of mares inseminated on a single occasion with 500, 25, or 5 x 10^6 progressively motile spermatozoa from 1 of 2 stallions. Sixty-one mares were randomly assigned to 1 of 3 treatments: Group 1 (n=20) were inseminated into the uterine body with 500 x 10^6 sperm (controls). Group 2 (n=21) and group 3 (n=20) were inseminated in the tip of the uterine horn ipsilateral to the preovulatory follicle with 25 or 5 x 10^6 sperm, respectively. Mares were administered cloprostenol (250µg i.m.) to induce luteolysis and monitored by ultrasonography every other day until a follicle ≥30mm was detected, and then daily until ovulation was detected. GnRH (deslorelin 2.2 mg, Ovuplant®, Fort Dodge, IA) was administered when the dominant follicle was ≥35mm. Mares were inseminated 34 (n=29) or 40 hours (n=32) after GnRH. Data from 22 mare cycles were excluded because they either ovulated prior to planned insemination (n=11), did not ovulate (n=3), or ovulated > 4 days after GnRH administration (n=8). Semen was collected and immediately diluted with a skim milk extender (EZ-Mixin, OF, Animal Reproduction Systems, Chino, CA) to either 25 x 10^6 or 5 x 10^6 motile sperm/ml. Mares were inseminated as described in Table 1. Mares receiving 1 ml were inseminated with a flexible plastic artificial insemination pipette (IMV, France), while mares receiving 0.2 ml were inseminated using a disposable implant gun (Veterinary Concepts, Green Valley, WI) containing a 0.5 ml straw. Different insemination pipettes were used to optimize delivery of the two different volumes. The location of pipettes within the uterus was confirmed by transrectal ultrasonography prior to semen deposition.

Pregnancy was determined by ultrasonography at 16 days after ovulation. Pregnancy rates were not different between stallions (P>0.05), so results from the two stallions were combined (Table 1). There was no difference in pregnancy rates between mares bred 34 vs. 40 hours after GnRH administration 19/29 (65%) and 18/32 (56%), respectively (P>0.1). Pregnancy rates decreased as the number of motile spermatozoa inseminated decreased. Breeding mares with 25 x 10^6 progressively motile sperm into the tip of the uterine horn resulted in a pregnancy rate of 57%.

Table 1. Day 16 Pregnancy Rates from a Single Insemination

No. of Motile Sperm	No. Pregnant/No. Bred	% Pregnant
500 x 10^6 in 20 ml	18/20	(90%)[a]
25 x 10^6 in 1 ml	12/21	(57%)[b]
5 x 10^6 in 1 ml	3/10	(30%)[b]
5 x 10^6 in 0.2 ml	4/10	(40%)[b]

[a,b] Values with different superscripts differ (P<0.05).

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Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa

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Keywords: horse; low dose insemination; sexed semen; frozen/thawed semen; equine

Summary

The objective of this experiment was to determine the effects of flow cytometric sorting and freezing on stallion sperm fertility. A 2 x 2 factorial design was used to delineate effects of flow sorting and freezing spermatozoa. Oestrus was synchronised (July–August) in 41 mares by administering 10 ml altrenogest (2.2 mg/ml) *per os* for 10 consecutive days, followed by 250 µg cloprostenol i.m. on Day 11. Ovulation was induced by administering 3000 iu hCG i.v. either 6 h (fresh spermatozoa) or 36 h (frozen/thawed spermatozoa) prior to insemination. Mares were assigned randomly to one of 4 sperm treatment groups. Semen was collected from 2 stallions with an artificial vagina and processed for each treatment. *Treatment 1* (n = 10 mare cycles) consisted of fresh, nonsorted spermatozoa and *Treatment 2* (n = 16 mare cycles) of fresh, flow sorted spermatozoa. Spermatozoa to be sorted were stained with Hoechst 33342 and sorted into X- and Y-chromosome-bearing populations based on DNA content using an SX MoFlo sperm sorter. *Treatment 3* (n = 16 mare cycles) consisted of frozen/thawed nonsorted spermatozoa (frozen at 33.5 x 10^6 sperm/ml in 0.25 ml straws) and *Treatment 4* (n = 15 mare cycles) of flow sorted frozen/thawed spermatozoa (frozen at 60.4 x 10^6 sperm/ml). Concentrations of sperm in both cryopreserved treatments were adjusted, based on predetermined average post-thaw motilities, so that each insemination contained approximately 5 x 10^6 motile spermatozoa. Hysteroscopic insemination of 5 x 10^6 motile spermatozoa in a volume of 250 µl was used for all treatments. Pregnancy was determined ultrasonographically 16 days postovulation.

No differences were found (P>0.1) in the pregnancy rates for mares inseminated with fresh nonsorted (4/10 = 40.0%), fresh flow sorted (6/16 = 37.5%), frozen/thawed nonsorted (6/16 = 37.5%) and flow sorted frozen/thawed spermatozoa (2/15 = 13.3%). Pregnancy rates tended (P = 0.12) to be lower following insemination of frozen/thawed flow sorted spermatozoa. Further studies are needed with a larger number of mares to determine if fertility of flow sorted frozen/thawed spermatozoa can be improved.

Introduction

A safe and reliable method for preconceptual sex selection of offspring has been sought for decades in man, livestock and companion animals. Johnson *et al.* (1989) were the first to report a reliable method to predetermine sex, by using DNA as a quantitative marker for X- and Y-chromosome-bearing spermatozoa and sorting spermatozoa via flow cytometry. This method was used subsequently to sort stallion spermatozoa and produce foals of predetermined sex (Buchanan *et al.* 2000; Schenk *et al.* 2000), but additional studies are required to make this technique applicable in the horse industry. Flow sorted stallion spermatozoa will be of limited use until methods for inseminating low numbers of spermatozoa are improved and until a successful cryopreservation technique is developed for flow sorted stallion spermatozoa.

Pickett and Voss (1975) determined that maximum fertility in mares is obtained by using a single insemination dose of 500 x 10^6 progressively motile spermatozoa (pms). At current rates at which stallion sperm can be sorted by flow cytometry, more than 5 days of continuous sorting would be required to accumulate 500 x 10^6 spermatozoa of each sex. Recently, several new techniques have been developed to increase fertility when using low numbers of stallion sperm. McCue *et al.* (2000) achieved a 21% pregnancy rate by surgically depositing 30,000 pms directly into the oviduct of pre-ovulatory mares. Munsaing *et al.* (1998) reported a 10% pregnancy rate when as few as 1 x 10^6 spermatozoa were deposited into the mare's oviduct through the uterotubal junction using a hysteroscopic technique. In a subsequent trial, a 30% pregnancy rate was reported from the insemination of 3.6 x 10^6 progressively motile spermatozoa placed on the uterotubal papilla with the use of an endoscope (Vazquez *et al.* 1998). Utilising a less invasive technique, Buchanan *et al.* (2000) inseminated 5 x 10^6 pms by use of an ultrasound guided method to direct a flexible insemination pipette to the tip of the uterine horn ipsilateral to the ovary containing the dominant follicle. The 35% pregnancy rate obtained was similar to that reported by Vazquez *et al.* (1998). Morris *et al.* (2000) used a videoendoscopic technique similar to that used by Bircher and Allen (1992) and achieved a 66% pregnancy rate when only 1 x 10^6 pms were placed onto and around the uterotubal papillae. Since this technique resulted in the highest reported pregnancy rates using low sperm numbers, we utilised videoendoscopic insemination in the present study to inseminate low numbers of flow sorted and nonsorted, fresh and frozen/thawed stallion spermatozoa.

Limited research has been reported on the fertility of flow sorted stallion sperm. The first pregnancy from flow sorted stallion spermatozoa was produced by surgical oviductal insemination (Schenk *et al.* 2000). The following season, Buchanan *et al.* (2000) reported a 60% pregnancy rate using 25 x 10^6 flow sorted stallion

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spermatozoa deposited deep into the uterine horn.

The ability to cryopreserve sorted spermatozoa would greatly increase the practicality of using flow sorted spermatozoa in the horse industry. Stallion sperm do not survive for long periods after the sorting process. Therefore, mares must be inseminated immediately after sperm sorting. However, if the sperm could be frozen following sorting, they could be used at any future time or location.

Several concerns should be addressed in the development of a cryopreservation protocol for use with flow sorted stallion spermatozoa. When using conventional techniques to freeze stallion spermatozoa, a single medium is not available that works best for all stallions (Squires *et al.* 1999). The same can be expected when freezing flow processed stallion spermatozoa but, as a means to decrease variables in studies of flow-sorted, frozen/thawed stallion spermatozoa, the freezing medium that produces the best results for most stallions should be identified and used.

Furthermore, it has been reported that spermatozoa from individual stallions can respond quite differently to cryopreservation (Muller 1987). Variation between stallions is also apparent in the ability of their sperm to be sorted by flow cytometry into X- and Y-chromosome-bearing populations. Because of these variations, it is necessary to carefully select stallions for this process that are most likely to survive the rigours of both flow sorting and cryopreservation.

Cryopreservation of flow sorted bull spermatozoa has been studied extensively. Seidel *et al.* (1999a) reported that the pregnancy rates for heifers inseminated with flow sorted frozen/thawed spermatozoa (182/353 = 51%) were similar to that for frozen/thawed nonsorted spermatozoa (2737/5 = 73%; P>0.05). Seidel *et al.* (1999b) also reported that pregnancy rates for heifers inseminated with low numbers of sorted, frozen/thawed sperm are generally within 90% of rates obtained with nonsorted, frozen/thawed sperm samples containing 7 to 20 times more spermatozoa per insemination.

In addition to developing techniques for inseminating low numbers of sperm for use with flow sorted spermatozoa, insemination with low numbers of nonsorted frozen/thawed spermatozoa is also of interest. This is particularly true for stallions with a limited inventory of frozen samples, for those that are dead or no longer capable of producing fresh spermatozoa, and for those that have poor sperm quality after cryopreservation.

The present study used a 2 x 2 factorial design to determine the effects of flow cytometric sex selection and cryopreservation of sperm on the fertilizing capacity of stallion spermatozoa. The objectives of this study were: 1) to determine if flow sorting stallion spermatozoa decreased pregnancy rates at an insemination dose of 5 x 10^6 prm; 2) to compare pregnancy rates of mares inseminated with low numbers of fresh sperm to those with frozen/thawed sperm; and 3) to determine whether pregnancies can be achieved when low numbers of flow sorted frozen/thawed stallion spermatozoa are inseminated into mares.

Materials and methods

A preliminary experiment was conducted to determine the preferred medium for cryopreservation and selection of stallions to be used in this study, as follows:

Semen collection and evaluation

Semen was collected from each of 7 stallions using a CSU model artificial vagina[1] equipped with an inline gel filter. After collection, the semen was evaluated for gel-free volume, motility and sperm concentration. The total ejaculate was extended at a ratio of 1:1 (v:v:mediu:semen, v/v) with pre-warmed HBGM-3 (adapted from Parrish *et al.* 1988) and centrifuged at room temperature for 15 min at 600 *g* to concentrate the spermatozoa and decrease seminal plasma concentration. After centrifugation, the supernatant containing 50% of the seminal plasma was removed, leaving a soft sperm pellet of ~5 ml. The post-centrifugation concentration (~1200 x 10^6 sperm/ml) was determined using the Densimeter[1].

Processing of control semen

An aliquot was taken from this sample and frozen using conventional freezing methods (Squires *et al.* 1999) for stallion spermatozoa (control). Sperm were extended in a final freezing concentration of 20 x 10^6 sperm/ml in room temperature lactose EDTA extender. Extended sperm were loaded into 0.25 ml straws and placed on a freezing rack at room temperature. The loaded freezing rack was then placed in static liquid nitrogen vapour at approximately -100°C, where straws were allowed 5 min to freeze, then plunged into liquid nitrogen for storage.

Processing of treated spermatozoa

The remaining sample was processed by a method which simulated preparation for flow sorting, however, the sperm were not processed through the MoFlo[2] instrument.

All treatment samples were processed identically until freezing. After centrifugation, the spermatozoa were extended to 400 x 10^6 sperm/ml in HBGM-3, in a total volume of 1 ml. Each sample was stained with 25 µl Hoechst 33342[3] (prepared in deionized water at 5 mg/ml) and incubated at 34°C for 1 h. Following incubation, samples were diluted to 160 x 10^6 sperm/ml with the addition of 3 ml pre-warmed HBGM-3 containing 2 ml/ml red food colouring (1% FD&C No. 40). Samples were then filtered through a 40 micron filter apparatus into a 6 ml polypropylene tube.

Samples were prepared in triplicate (3 potential freezing extenders) with sheath fluid (HBGM-3). Sperm were diluted in sheath fluid and freezing extender without glycerol (representing each fluid) at 600,000 sperm/ml. In a 50 ml Falcon tube, 150 µl of sperm sample was combined with 21 ml sheath fluid and 4 ml of each of 3 freezing extenders (lactose EDTA, FR3 and COS; Squires *et al.* 1999). Samples were incubated for 2 h at ambient temperature to simulate the amount of time that would be needed for sorting of X- and Y-chromosome-bearing populations. Samples were then reconcentrated for freezing. Tubes were centrifuged (22°C) for 20 min at 850 *g* and the supernatant was aspirated, leaving a 200 µl sperm pellet. Based on 85% recovery rates, each pellet contained 12.75 x 10^6 sperm. To reach the desired freezing concentration of 20 x 10^6 sperm/ml, 0.64 ml of the appropriate freezing extender (with glycerol) was added to each pellet. Spermatozoa were then frozen (~8 h post-collection) according to the appropriate protocol for each extender (Squires *et al.* 1999).

Freezing of treated spermatozoa

For samples in FR3 and COS, the 50 ml tubes containing the extended pellets were suspended in a 600 ml beaker of 22°C water, covered, placed in a 5°C cold room and allowed to cool for

90 min (-2°C/min). Samples were then gently vortexed to redistribute spermatozoa in the medium, and 0.25 ml was loaded in each precooled 0.25 ml straw. Loaded straws were placed on a precooled freezing rack, which was then placed in static liquid nitrogen vapour at approximately -100°C. After 5 min in vapour, straws were plunged into liquid nitrogen for storage.

For samples in lactose EDTA, sperm were loaded at ambient temperature (22°C) in 0.25 ml straws and frozen in a similar way to control samples.

Thawing and evaluation

To thaw sperm, straws were placed in a warm water bath (37°C) for 30 s. The end of the straw containing the stainless steel ball was cut and sperm expelled into prewarmed 1.5 ml polypropylene eppidues. Two straws/ejaculate/treatment were evaluated.

Sperm quality was determined based on post-thaw motility. Visual motility for all samples were read by 2 technicians at 0.5 and 2 h post-thaw. Each sample was evaluated for total and progressive motility by each technician, and readings were averaged between technicians.

Treatment differences ($P<0.05$) with respect to total and progressive motility at 0.5 and 2 h were detected using Analysis of Variance in SAS. Treatment means were separated using Tukey's Studentised Range (HSD) test in the General Linear Models Procedure.

The results from this preliminary experiment were examined, and 2 stallions whose sperm exhibited acceptable post-thaw motility (>35% prog) were selected for use in the next experiment. Additionally, FR5 was chosen as the preferred freezing extender for use in this experiment, since the post-thaw motilities were generally greater for spermatozoa frozen in this extender.

Semen collection and processing

Semen was collected from each of two 4-year-old Arabian stallions on alternating days throughout the duration of the project using a CSU model artificial vagina equipped with an in-line gel filter. After collection, the gel-free volume, motility and spermatozoal concentration for each ejaculate was determined. The semen was then extended (1:1) (extender:semen) with prewarmed HBGM-3 (adapted from Parrish *et al.* 1988) and centrifuged immediately at ambient temperature for 15 min at 400 *g* to concentrate the spermatozoa and remove 90% of the seminal plasma. After centrifugation, the supernatant was removed, leaving soft sperm pellets with sperm concentrations of >1.2 x 10^9/ml. The pellets were transported immediately to another laboratory (~5 min) for further processing in one of 4 treatment groups.

Treatment 1: Mares (n = 10 cycles) were inseminated 6 h post-hCG administration with 5 x 10^6 fresh, nonsorted prep via hysteroscopic insemination. Following centrifugation, sperm were resuspended in the *Sorb* in HBGM-3 at ambient temperature and at a concentration of ~1.2 x 10^9 sperm/ml for approximately 6 h (to simulate the time needed to sort spermatozoa for *Treatments 2* and *4*).

Prior to insemination, motility was visually evaluated, concentration was determined using a haemacytometer, and a 250 µl dose containing 5 x 10^6 pmi was prepared in a skim milk + egg yolk extender (FR4) and inseminated immediately.

Treatment 2: Mares (n = 16 cycles) were inseminated 6 h post-hCG administration with fresh, flow sorted spermatozoa via hysteroscopic insemination. The concentration of sperm in the soft pellet was determined with the Densimeter, and a volume of HEPES BGM-3 was added to bring the spermatozoal concentration to 400 x 10^6 sperm/ml. A stock solution of 8.89 mmol/l Hoechst 33342, a stain that binds to adenine-thymine-rich regions of the minor groove of the DNA helix, was prepared in sterilpure water (Johnson *et al.* 1989). One ml sperm suspension was stained with 25 µl Hoechst 33342 and incubated at 34°C for 1 h. The stained samples were then diluted to 100 x 10^6 sperm/ml for sorting with the addition of 5 ml HEPES BGM-3 containing red food colouring (2 µl/ml of 1% FD&C No. 40). The samples were filtered at unit gravity through a 40 µ nylon mesh filter into 6 ml polypropylene tubes to remove any debris and clumped spermatozoa and held at ambient temperature until they were analysed and sorted.

Spermatozoa were sorted using a Cytomation SX MoFlo flow cytomercial sorter modified for sperm sorting. Argon lasers, emitting 150 mW at wavelengths of 351 and 364 nm, were used on each of 2 MoFlo instruments at 50 psi. HEPES BGM-3 prepared without BSA was used as the sheath fluid (pH = 7.2, 300±310 mOsm). Approximately 1,000 live sperm/s were sorted and collected into 50 ml centrifuge tubes containing 4 ml FR4. Tubes containing spermatozoa of corresponding sex were pooled from each flow cytometer, and sorted spermatozoa were centrifuged for 30 min at 850 *g* at 25°C. The supernatant was removed, leaving a pellet of approximately 100 µl, and the pellets then resuspended in 1500 µl room temperature FR4 and gently mixed. The spermatozoal concentration was then determined using haemocytometer counts (n = 4) and the percentage of motile sperm in the sorted samples (X and Y) was evaluated visually. Additional FR4 was then added to each sample to obtain the desired final spermatozoal concentration of 21.7 x 10^6 pmi/ml. The predetermined volume (230 µl) containing 5 x 10^6 motile spermatozoa was then loaded into an equine OPU catheter[4] and inseminated using the hysteroscopic insemination technique (Morris *et al.* 2000).

Treatment 3: Mares (n = 16 cycles) were inseminated 30 h post-hCG administration with 5 x 10^6 nonsexed motile frozen-thawed spermatozoa via hysteroscopic insemination. After initial processing, the post-centrifugation concentration was determined using the Densimeter, and pellets were adjusted to their final concentration of 33.5 x 10^6 sperm/ml in a skim milk – egg yolk + 4% glycerol extender (FR5). Sperm suspensions were protected from light and held in room temperature until samples from *Treatment 4* were ready to freeze. Sealed tubes containing the sperm pellets were suspended in 600 ml beakers containing room temperature water, covered and placed in a 5°C cold room, where sperm pellets were allowed to cool slowly (-0.2°C/min) to 5°C. After 90 min, sperm were packaged into 0.25 ml polyvinylchloride straws, set on a rack and frozen in static liquid nitrogen vapour.

The contents of each straw comprised one insemination dose of 230 µl containing approximately 5 x 10^6 motile spermatozoa (average post-thaw motility = 65%). Each straw was thawed in a 37°C water bath for 30 s, the contents transferred to a prewarmed 6 ml falcon tube, and then drawn into an equine OPU catheter for hysteroscopic insemination (Morris *et al.* 2000).

Treatment 4: Mares (n = 16 cycles) were inseminated 30 h post-hCG administration with 5 x 10^6 motile sperm that had been

TABLE 1: Percentage of motile spermatozoa observed post-thaw of control and flow processed stallion spermatozoa frozen in 5 extenders

Extender	Total motility (0.5 h)	Progressive motility (0.5 h)	Total motility (2 h)	Progressive motility (2 h)
Control (lactose EDTA)	39[a]	34[a]	24[a]	20[a]
FR 5	28[b]	22[b]	19[b]	14[b]
Lactose EDTA	22[c]	19[b]	14[c]	8[c]
OC 5	22[c]	12[c]	7[c]	4[c]
s.e.m.*	1.58	1.93	1.08	0.86

[a,b,c]Values within columns with similar superscripts do not differ (P>0.01). *s.e.m. was calculated by taking the square root of error term of ANOVA(m).

flow sorted and then frozen and thawed. Sperm pellets for this treatment were treated identically to those in *Treatment 2* throughout the sorting process. Spermatozoa were sorted using SX MoFlo flow cytometer/sperm sorters and collected in 50 ml centrifuge tubes containing 4 ml FR4. Tubes containing spermatozoa of corresponding sex were pooled from each flow cytometer, and sorted spermatozoa were centrifuged at 22°C for 20 min at 850 *g*. The supernatant was removed, leaving a pellet of approximately 100 µl, and this pellet then resuspended in 400 µl FR5. Sealed tubes containing the sperm pellets were suspended in 600 ml beakers containing room temperature water, covered and placed in a 5°C cold room, where sperm pellets were allowed to cool slowly (-0.3°C/min) to 5°C over a 90 min period. Haemacytometer counts (n = 4) were performed to determine the post-centrifugation concentration, and a volume of FR5 was then added to each tube at 5°C to obtain a final concentration of 64.4×10^6 sperm/ml. Sperm samples were warmed gently, loaded into 0.25 ml polyvinylchloride straws, and frozen as for *Treatment 3*.

The contents of each straw comprised one insemination dose of 230 µl which contained approximately 5×10^6 motile spermatozoa (average post-thaw motility = 35%). Each straw was thawed in a 37°C water bath for 30 s, the contents transferred to a 6 ml tube and then loaded into an equine GIFT catheter for intercalate hysterosalpingo insemination (Morris *et al*, 2000).

Mare management:

Forty-one mares of light-horse type, age 3-16 years, were synchronised by administering alfrenogest (Regumate)[5] (0.2 *mg/ml per os; 10 ml/horse/day*) for 10 consecutive days, followed by an injection of cloprosterol (Estrumate)[6] (250 µg, i.m.) on Day 11. After cloprosterol administration, mares were examined via rectal palpation and ultrasonography once every other day until a follicle ≥ 30 mm diameter was detected. Mares with large follicles (≥ 50 mm) were examined each morning until a follicle ≥ 55 x 25 mm was detected. These mares were immediately administered 3000 iu human chorionic gonadotropic (Chorulon)[7] (hCG) and assigned randomly to one of the 4 treatment groups. Mares were inseminated with fresh sperm 6 h post-hCG, and with frozen/thawed sperm 30 h post-hCG.

All mares were inseminated hysteroscopically (Bracher and Allen 1992, Morris *et al*, 2000) with a total volume of 230 µl. Briefly, the sperm were loaded into an equine GIFT catheter using a 6 ml disposable syringe enclosed on the injection port on the distal end of the catheter. The loaded catheter was drawn into an outer polypropylene cannula, which was then passed down the working channel of a Pentax gallative EC3430F endoscope[8]. The flexible endoscope (1.6 m long with an outer diameter of 12 mm) was guided through the cervix and filtered air was introduced into the uterus to facilitate passage of the instrument through the uterine lumen. With the aid of a video monitor, inseminators directed the endoscope through the lumen of the uterine horn ipsilateral to the ovary containing the preovulatory follicle. When the tip of the endoscope came to within 5-8 cm of the papilla of the uterotubal junction, the GIFT catheter containing the sperm suspension was extruded from the working channel of the endoscope and placed against the papilla. The plunger of the syringe was then depressed, depositing the small volume of inseminate on and around the surface of the papilla. The endoscope was then withdrawn steadily from the uterus while simultaneously evacuating the filtered air from the uterine lumen.

All mares were inseminated only once, on the side ipsilateral to impending ovulation. To determine the day of ovulation, mares were examined using ultrasound daily after insemination until ovulation was detected. Pregnancy examinations were performed ultrasonographically on Days 12, 14, 16, 25 and 33 after ovulation (day of ovulation = 0). Pregnancy status was determined based upon Day 16 examination. Eight mares that became pregnant after insemination with flow sorted sperm were allowed to foal to determine the normality of offspring resulting from flow sorted spermatozoa, as well as to confirm the resulting sex of the foals.

Chi-square analysis was used to test for differences ($P<0.05$) in the fertilising ability of spermatozoa in the 4 treatments.

Results

The results of the preliminary experiment comparing various extenders for cryopreservation are presented in Table 1. Post-thaw motilities of spermatozoa frozen by the control method were higher than motilities observed for all other treatments. Based on visual estimates of the percentage of motile sperm, FR5 was the preferred extender for cryopreservation of flow processed stallion spermatozoa. Spermatozoa that were processed for flow cytometry and subsequently frozen in FR5 exhibited the greatest percentage of motile spermatozoa post-thaw at 0.5 h (28%, $P<0.05$), as well as the highest percentage of progressively motile sperm at 2 h (14%; $P<0.05$) (Table 1).

Substantial variation was detected among the stallions used in the preliminary experiment, as detected by post-thaw

TABLE 2: Variation among stallions in the percentage of total and motile spermatozoa post-thaw, averaged across 3 freezing extenders

Stallion	Total motility (0.5 h)	Progressive motility (0.5 h)
A	41[a]	20[a]
B	38[a]	32[a]
C	38[a]	28[a]
D	24[b]	20[b]
E	23[b]	16[b,c]
F	17[c]	12[c,d]
G	13[c]	10[d]
s.e.m.*	2.0%	2.0%

[a,b,c,d]Values within columns with similar superscripts do not differ ($P>0.1$); *s.e.m. was calculated by taking the square root of (error terms of ANOVA / n).

modalities of control and flow processed spermatozoa. Post-thaw motilities for the 7 stallions are shown in Table 2. Total post-thaw motilities (0.5 h) ranged from 41–13%, and progressive post-thaw motilities (0.5 h) were 32–10%. Motilities observed at 2 h post-thaw are not presented, but they followed a similar trend as did motilities read at 0.5 h. Among stallions, total motilities (2 h) ranged from 30% (Stallion B) to 7% (Stallion F), and progressive motilities (2 h) from 24% (Stallion B) to 4% (Stallion F).

In the present trial, pregnancy rates were not different between stallions (Stallion A = 9/28, 32%; Stallion B = 9/29, 31%; $P>0.1$); therefore, fertility data were combined. No differences were found in the pregnancy rates of mares inseminated with fresh nonsorted, fresh flow sorted, frozen nonsorted and flow sorted frozen/thawed spermatozoa (Table 3). There was a tendency for pregnancy rates to be lower following insemination of flow sorted frozen/thawed spermatozoa when compared to all other treatments (13 vs. 36%; $P = 0.11$).

The mean diameter of the preovulatory follicle at the time of hCG treatment was 37.1 mm, range 33.5–45.0 mm. Ovulation was detected on Day 2 after hCG treatment in 95% of the mares, range 1–4 days.

One mare that became pregnant from insemination of flow sorted, frozen/thawed spermatozoa aborted the fetus at 246 days gestation. The fetus was of the correct predetermined sex. All of the remaining mares that became pregnant with flow sorted sperm were allowed to foal and the sex of 6 of the 7 foals corresponded correctly to the spermatozoa used. Therefore, of the 8 mares impregnated with sex-selected sperm, 7 foals (88%) were of the correct predetermined sex (Table 3).

Discussion

Upon comparison of freezing extenders in the preliminary experiment, FR3 was found to be the most desirable based on post-thaw motility of flow processed stallion spermatozoa. It should be realised, however, that FR3 may not be the preferred freezing extender for all stallions. For instance, spermatozoa collected and processed from Stallion F exhibited higher ($P<0.05$) progressive post-thaw motility when frozen in lactose EDTA (12%), than in FR3 (4%). Prior to application of this technology for individual stallions, it is recommended that each particular stallion be evaluated in each extender as a means of optimising potential fertilising capacity.

In previous studies of frozen stallion spermatozoa, great variation among stallions regarding the freezing ability of semen has been observed repeatedly. Pickett and Amann (1993) estimated that 25–30% of stallions produce semen that cryopreserves well, 35–50% produce semen that cryopreserves moderately and 25–40% semen that cryopreserves poorly. Similar results were obtained in the present trial with flow processed spermatozoa, except post-thaw motilities were even further depressed.

For commercial application of flow sorted frozen/thawed stallion spermatozoa, total post-thaw motilities >30% are desirable. Based on the present study, 3 of 7 stallions (42%) would qualify as candidates for future flow sorting applications. Stallions A and C were chosen to be used in the present study to compare pregnancy rates of mares inseminated with low numbers of flow sorted frozen/thawed stallion spermatozoa. Additionally, due to higher post-thaw motilities exhibited by sperm frozen in FR3, this extender was chosen as the cryopreservation medium to be used in the present experiment.

The recommended insemination dose to achieve maximum fertility in mares, as suggested by Pickett and Voss (1975), remains as 500×10^6 pms inseminated every other day while the mare is in oestrus. However, in several studies, no decrease in fertility has been found when researchers used only 100×10^6 pms (Pickett *et al.* 1974; Demick *et al.* 1976), although inseminating 50×10^6 pms did reduce pregnancy rates (Pickett *et al.* 1976). Due to the fact that only limited numbers of spermatozoa are available following flow sorting, and many of these may have compromised function, it is imperative that insemination techniques be developed that permit low numbers of spermatozoa to be inseminated into mares without reducing fertility.

Perhaps the most exciting finding of the present experiment is the 38% pregnancy rate achieved after insemination of only 5×10^6 motile frozen/thawed spermatozoa. Vidament *et al.* (1997) and Leipold *et al.* (1998) estimated that approximately 300×10^6 pms in the optimum insemination dose for frozen/thawed stallion spermatozoa to be used for insemination, and pregnancy rates of

TABLE 3: Pregnancy rates from a single insemination of nonsorted and flow sorted, fresh and frozen/thawed equine spermatozoa

Treatment	Mares inseminated	Mares pregnant Day 16 [% pregnant]	Sperm inseminated ♀	Sperm inseminated ♂	Resulting sex (foal) ♀	Resulting sex (foal) ♂
Nonsorted fresh	10	4 (40)	--	--	--	--
Flow sorted fresh	16	6 (38)	4	2	4	2
Nonsorted frozen	16	8 (50)	--	--	--	--
Flow sorted frozen	16	2 (13)	2	--	1[a]	1

[a]The mare lost her pregnancy at ~8 months; the fetus was male.

20 and 40% per cycle were achieved, respectively. The number of spermatozoa inseminated in the present study was less than 2% of this recommended minimum number of sperm, but similar pregnancy rates were achieved (36%). The success in the present study can be attributed, in part, to the use of videoendoscopic insemination, which permitted placement of the sperm directly onto the papilla of the uterotubal junction.

The significance of these results achieved using low numbers of nonsorted frozen/thawed spermatozoa should not be underestimated by the equine industry. With the ability to use only 5×10^6 motile spermatozoa per insemination, current frozen semen inventories could be used to inseminate increased numbers of mares. Stallions labelled as 'poor freezers' may also benefit from this insemination method. These benefits are more difficult to predict, however, as the cause of these 'poor freezers' is not yet fully understood.

The lack of a significant difference between the pregnancy rates obtained from the use of nonsorted vs. flow sorted spermatozoa is in agreement with previous studies in our laboratory (Buchanan et al. 2000). It is realised, however, that additional studies with larger numbers of mares are necessary to detect true differences in fertility of flow sorted stallion spermatozoa.

It is surprising that the pregnancy rates of flow sorted spermatozoa could equal that of nonsorted spermatozoa, since the procedure is a lengthy and highly insulting process. Prior to sorting, spermatozoa are incubated for 1 h at 34°C with Hoechst 33342. During sorting, spermatozoa are pumped at high pressure through fine tubing at ~100 km/h and are then stored for several hours diluted at 500,000 sperm/ml. Any of these steps could potentially induce harm, but it is not yet clear at which point the greatest amount of damage occurs.

For this study, the appropriate nonsorted controls were those in which spermatozoa did not undergo any of the potentially harmful treatments necessary in the sperm preparation and sorting process. Although the spermatozoa for these controls did not endure any of the necessary processing steps, the time interval from collection to insemination (or freezing) remained constant for all treatment groups. Additionally, for both frozen treatment groups, the number of motile spermatozoa to be inseminated, along with the volume of the inseminate, were the most critical factors for evaluation of the process. Therefore, due to differences in the post-thaw motilities of sorted vs. nonsorted spermatozoa, the concentrations of control and sorted sperm that were cryopreserved were not identical.

Timing of insemination in relation to hCG administration was different for fresh spermatozoa vs. frozen spermatozoa. Similar to previous work with inseminations at the uterotubal junction (Morris et al. 2000), hCG was administered a maximum of 8 h prior to insemination for all fresh sperm treatments (1 and 2). When using frozen/thawed spermatozoa, it has been shown that pregnancy rates are highest when insemination occurs within 12 h prior to ovulation (Amann and Pickett 1987). Therefore, spermatozoa from both frozen treatments (3 and 4) were inseminated 30 to 32 h after hCG administration. In retrospect, it might also be beneficial to inseminate fresh flow sorted spermatozoa 24 to 30 h after hCG administration so that spermatozoa were in the mare within 12 to 24 h of ovulation. In previous studies of flow sorted bull spermatozoa, it was observed that sorted sperm exhibited a higher proportion of membranes that were acrosome-reacted or precapacitated (Maxwell et al. 1998). Therefore, it may be that flow sorted spermatozoa are similar to frozen/thawed spermatozoa and have limited longevity in the oviduct. Pregnancy rates may be maximised, therefore, by inseminating flow sorted stallion spermatozoa within 12 h of ovulation. Timing of insemination of flow sorted stallion spermatozoa warrants additional study.

The pregnancies obtained by insemination of only 5×10^6 motile flow-sorted, frozen/thawed spermatozoa are of great interest. Although the pregnancy rates for this treatment group were low (2/15; 13%), this is the first report of pregnancies obtained from the insemination of flow sorted cryopreserved stallion spermatozoa. When comparing the results from the 4 treatments, it appears that damage to stallion spermatozoa induced by flow sorting is additional to that caused by freezing and thawing. This is evident when comparing the pregnancy rates of flow sorted frozen/thawed spermatozoa (13%) to that of nonsorted frozen spermatozoa (36%) or fresh flow sorted spermatozoa (38%). These differences would probably be greater if larger numbers of mares were used. It is imperative that a less harmful method of freezing stallion spermatozoa be developed if flow sorted frozen/thawed spermatozoa is to become practical.

The cause of the late term abortion in one mare was not known. A phenotypically normal fetus was recovered from the mare. Necropsy of the fetus reflected normal development to the time of pregnancy loss. Blood samples recovered from the mare and fetus were analysed and no abnormalities were detected. There was no evidence that the abortion was related to the use of flow-sorted spermatozoa.

In summary, we have demonstrated, for the first time, that pregnancies in mares can be obtained using only 5×10^6 flow sorted, frozen/thawed stallion spermatozoa when sperm are placed on and around the uterotubal junction by hysteroscopic insemination. Furthermore, hysteroscopic insemination can be used effectively to inseminate low numbers of frozen/thawed stallion spermatozoa as well as flow sorted stallion spermatozoa, and reasonable pregnancy rates can be achieved.

Manufacturers' addresses

[1]Animal Reproduction Systems, Chino, California, USA.
[2]Cytomation Inc., Fort Collins, Colorado, USA.
[3]ICN Biochemicals Inc., Aurora, Ohio, USA.
[4]Cook Veterinary Products, Brisbane, Queensland, Australia.
[5]Interved Inc., Delaware, Ohio, USA.
[6]Bayer Corporation, Agriculture Division, Shawnee Mission, Kansas, USA.
[7]Pentax, Orangeburg, New York, USA.

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